TITLE: Teneurin C-Terminal Associated Peptides (TCAP) and Methods and Uses Thereof

RELATED APPLICATIONS

This application claims the benefit and priority of United States provisional patent application number, US 60/377,231, Filed May 3, 2002, entitled "Teneurin C-Terminal Associated Peptides (TCAP)" and US 60/42,4016, filed November 6, 2002, entitled "Method for Modulating Stress using Teneurin C-Terminal Associated Peptide-1(TCAP-1)". This application also claims priority from United States provisional patent application number, US 60/ 376,879, filed May 2, 2002, entitled, " Immortalized Hypothalamic Neuronal Cell Lines ". All of these references are incorporated in their entirety by reference.

FIELD OF THE INVENTION

The invention relates to a novel family of peptides associated with the c-terminal region of the teneurin molecule, to a nucleic acid molecule encoding said peptides and to methods and uses therefore.

BACKGROUND OF THE INVENTION

The aetiology of any neuropathology is a complex interplay of genetic, physiological and environmental factors. Effective treatment of these conditions will ultimately depend upon the understanding of the cognate genes and their products. In recent years, it has become apparent that large families of related genes are responsible for the regulation of neuropathologies involving anxiogenic peptides. The identification and characterization of these gene families and how they interact is an essential step towards ultimately effectively treating the pathology. The aberrant regulation of neuronal growth can manifest as a variety of pathological conditions depending upon the age. Deficits in neuronal growth in foetal or neonatal animals can cause such diseases as learning deficits, mental retardation, autism, or schizophrenia. At later ages in juvenile individuals it may manifest as affective disorders such as panic disorder, depression, anorexia nervosa, obsessive-compulsive disorder later in adults. In adults

such neuronal growth problems could lead to neurodegenerative illnesses such as Alzheimer's Disease or Parkinsons's Disease.

The onset of mood disorders, such as depression or post traumatic stress disorder, involve the altered function of multiple loci in the brain that regulate emotionality, memory and motivation (Manji et al., 2001; Drevets, 2001; Nestler et al., 2002). However, many of the cellular signaling molecules that mediate communication within and between these regions are unknown, leading to an incomplete understanding of the origin of such disorders.

Many neuropeptides show the presence of three or four paralogous structures as evidenced by the neuropeptide Y (NPY) (Larhammar, 1996a,b), proopiomelanocortin (POMC) (Danielson, 2000) and recently, the corticotropin releasing factor (CRF) family (Vale et al., 1981, Vaughan et al., 1995; Lovejoy and Balment, 1999; Lewis et al., 2001 Reyes et al., 2001; Hsu and Hseuh, 2001).

A family of neuronal cell surface proteins has been identified that are predominantly expressed in the nervous system. These proteins have been named teneurins (Rubin et al, Developmental Biology 216, 195-209 (1999)). Four basic teneurins have been identified Ten M1, Ten M2, Ten M3, and Ten M4. The Ten-M or Odz proteins were originally discovered in Drosophilia (Levine et al., 1994; Baumgartner et al., 1994) and are presently the only known example of a pair-rule gene that is not a transcription factor. The Ten-M gene is initially activated during the blastoderm stage, then down regulated before being expressed at later stages. The highest levels of Ten-M occur in the central nervous system where the protein occurs preferentially on the surface of axons (Levine et al., 1994; Levine et al, 1997). Mutations of the Ten-M/Odz gene result in embryonic lethality (Baumgartner et al., 1994; Levine et al., 1994).

Four Ten-M paralogous genes, called Teneurins, exist in vertebrates and encode a Type II transmembrane protein where the carboxy terminus of the protein is displayed on the extracellular face of the cell (Oohashi et al., 1999). The teneurin proteins are about 2800 amino acids long. There is a short stretch of hydrophobic residues at 300 to 400 amino acids after the

amino terminus that appear to act as the membrane spanning site. In the cytoplasmic N-terminal portion, is a conserved proline-rich SH3-binding site indicating a potential site where by they bind other proteins. Evidence suggests that the protein may be cleaved from the membrane at a Furin-like cleavage motif (RERR) located around residue 528 in teneurin 2 (Rubin et al., 1999). However, this motif is not present in the other paralogues and therefore a soluble version of the protein may not occur for all paralogues. There are a series of cysteine-rich EGF-like repeats carboxy terminal to this. Homodimerization occurs between Ten M1 forms via interaction between EGF-like modules 2 and 5 (Oohashi et al., 1999).

The ten-m gene appears to be upregulated by stressors. Wang et al (1998) showed that a ten-M like transcript, named DOC4 (downstream of chop) in mammalian cells was upregulated by the transcription factor GADD153/CHOP. This transcription factor is induced by several types of cellular stressors including UV light, alkylating agents or conditions triggering endoplasmic reticulum (ER) stress responses, such as, deprivation of oxygen, glucose or amino acids, or interference of calcium flux across the ER membrane (Zinszner et al, 1998). GADD153 is a small nuclear protein that dimerizes with members of the C/EBP family of transcription factors (Ron and Habener, 1992). It does not appear to homodimerize. GADD153 undergoes a stressor inducible phosphorylation by a p38-type MAP kinase which also enhances the transcriptional activation of GADD153 (Wang et al., 1996). High expressions of GADD153 will lead to cell cycle arrest (Zhan et al. 1994). These studies suggest that the teneurin gene may play a significant role in the regulation of the stress response of neurons and other cells.

Overexpression of teneurin 2 into the mouse neuroblastoma cells (Nb2a) augmented the amount of neurite outgrowth and a tendency to enlarge the growth cones. The number of filamentous actin-containing filopodia was also enhanced in the teneurin 2 overexpressing cells (Rubin et al., 1999). The expression of the teneurin genes have been examined in embryonic zebrafish (Mieda et al, 1999), chicken (Rubin et al., 1999) and mouse (Ben-Zur et al., 2000) although their expression patterns have not been finely resolved. The

transcripts are found in a number of peripheral tissues but are found predominantly in the central nervous system. In the embryonic chicken brain, teneurin 1 and 2 are expressed in the retina, telencephalon, the optic tectum and the diencephalons. The mRNA for teneurin 1 was found mainly in the intermediate zone of the dorsal thalamus whereas teneurin 2 was found in the intermediate zone of the thalamus (Rubin et al., 1999). In zebrafish, teneurin 4 is faintly expressed throughout gastrulation, although there is no teneurin 3 expression. Teneurin 3 expression begins at the notochord and the somite around the tailbud stage. In later stages (14 h post fertilization), teneurin 3 is expressed in the somites, notochord and brain while teneurin 4 expression was confined to the brain. Teneurin 3 becomes defined within the optic vesicles and region covering the caudal diencephalons and mesencephalon with the expression strongest in the anterior mesencephalon. Teneurin 4 has its strongest expression toward the midbrain hindbrain border. By 23 h post fertilization, teneurin 3 is expressed in the dorsal part of the tectal primordium and the ventral midbrain while teneurin 4 is expressed in the ventral primordium (Mieda et al., 1999).

Neuropathological conditions tend to be complex and not very well understood. As such, there is a need to better understand the mechanisms involved and to develop a method of diagnosis and treatment of said conditions. There is also a need for the identification and design of therapeutic compounds for said conditions.

SUMMARY OF THE INVENTION

The present invention provides a teneurin c-terminal associated peptide (TCAP), existing as a 40 - 41-residue sequence on the c-terminal exon of Ten- M 1, 2, 3, or 4 that is correspondingly named TCAP 1, 2, 3, and 4. In another embodiment, the invention provides a peptide that has the amino acid sequence consisting of a 40- or 41 amino acid sequence located at the c-terminus of the teneurin 1-4 peptides, to analogs, species homologues, dervivatives, variants, allelic variants, to sequences having substantial sequence identity thereto and to obvious chemical equivalents thereto. In

another embodiment the TCAP peptides of the invention can further include an amidation signal sequence at the carboxy terminus (hereinafter referred to as "preTCAP"). Such amidation signal amino acid sequence can include but is not limited to GKR and GRR. The invention also provides fusion proteins comprising the TCAP peptides noted above, to labeled TCAP Peptides and to peptides comprising flanking amino acid sequence of 1-10 amino acids.

In one embodiment, the invention provides a TCAP peptide that has neuronal communication activity. In another embodiment the invention provides a TCAP peptide, an analog, derivative, variant, homolog that has similar activity. In one embodiment, the activity is neuronal communication. In another embodiment it is inhibition of cell proliferation, In yet another embodiment it is modulation of a stress response.

In one embodiment the TCAP sequence is a rainbow trout, zebrafish, human, mouse, *G. gallus*, or *D. melanogaster* TCAP. In another embodiment, the TCAP sequence comprises or consists of SEQ. ID. NOS: 13, 14, 21, 22, 29, 30, 37, 38, 45, 46, 53, 54, 61, 62, 69, 70, 77, 78, 85, 86, 93, 94, 101, 103 In yet another embodiment, the TCAP is a mouse or human TCAP. In one embodiment the TCAP has one of the sequence selected from the group consisting of SEQ. ID. NOS: 69, 70, 77, 78, 85, 86, 93, 94 (human) or SEQ. ID. NOS: 37, 38, 45, 46, 53, 54, 61, 62, (mouse).

In one aspect, the invention provides a TCAP consisting of any one of the SEQ. ID. NOS. noted above and an amidation signal sequence at the carboxy terminus. Preferably the amidation signal sequence is selected from the group consisting of GRR or GKR, such as, 15, 16, 23, 24, 31, 32, 39, 40, 47, 48, 55, 56, 63, 64, 71, 72, 79, 80, 97, 88, 95, 96.

Another aspect of the invention relates to an isolated teneurin c-terminal associated peptide that has the amino acid sequence as shown in SEQ. ID. NOS: 13, 14, 21, 22, 29, 30, 37, 38, 45, 46, 53, 54, 61, 62, 69, 70, 77, 78, 85, 86, 93, 94, 101, 103;or a fragment, analog, homolog, derivative or mimetic thereof. In a preferred embodiment, the TCAP peptides of the invention have anxiogenic activity. The invention also encompasses an antibody that can bind a TCAP peptide of the invention.

In another embodiment, the peptide of the invention is a TCAP mouse peptide having the amino acid sequence of: SEQ. ID. NOS: 37, 38, 45, 46, 53, 54, 61, 62.

In another embodiment, the peptide of the invention is a TCAP human peptide having the amino acid sequence of SEQ. ID. NOS: 69, 70, 77, 78, 85, 86, 93, or 94.

In another embodiment the peptides TCAP human and mouse peptides have an amidation signal sequence at the C-terminus.

In another embodiment, the peptide of the invention is a TCAP-1 and has the amino acid sequence of SEQ. ID. NOS.: 37,38, 69 or 70.

In another embodiment, the peptide of the invention is a TCAP-2 and has the amino acid sequence of SEQ. ID. NOS.: 46, 47, 77, or 78.

In another embodiment, the peptide of the invention is a TCAP-3 and has the following amino acid sequence motif:

QLLSXaa$_1$Xaa$_2$KVXaa$_3$GYDGYYVLSXaa$_4$EQYPELADSANNXaa$_5$QFL RQSEI (SEQ. ID. NO:135) ,

where Xaa$_1$ is G, S, or A; Xaa$_2$ is G or R; Xaa$_3$ is L or Q; Xaa$_4$ and Xaa$_5$ are independently V or I. In one embodiment, the TCAP-3 is a human or mouse TCAP- 3. In another embodiment, the TCAP- 3 has SEQ. ID. NO: 85, 86, 53, or 54. In another embodiment, the TCAP 3 sequence is SEQ. ID. NO.: 13, 14, 21 or 22.

In another embodiment, the peptide of the invention is a TCAP-4 and has the amino acid sequence SEQ. ID. NOS.: 29, 30, 61, 62, 93, or 94.

In another embodiment the peptides TCAP 1 to TCAP 4 have an amidation signal sequence at the C-terminus.

In yet another embodiment, the present invention provides as isolated nucleic acid molecule encoding a teneurin c-terminal associated peptide (TCAP) of the invention, as noted herein. In yet another embodiment, the isolated nucleic acid molecule of the invention consists of:

(a) a nucleic acid sequence as shown in SEQ.ID.NOS.: 18-20, 25-28, 33-36, 41-44, 49-52, 57-60, 65-68, 73-76, 81-84, 89-92, 97-100 or that wherein T can also be U or that encodes a peptide having an amino acid

sequence selected from the group consisting of : SEQ. ID. NOS: 13, 14, 21, 22, 29, 30, 37, 38, 45, 46, 53, 54, 61, 62, 69, 70, 77, 78, 85, 86, 93, 94, 101, 103 or that further has an amidation signal sequence (preferably GKR or GRR), at the carboxy terminus of said peptides, such as 15, 16, 23, 24, 31, 32, 39, 40, 47, 48, 55, 56, 63, 64, 71, 72, 79, 80, 97, 88, 95, 96;

(b) a nucleic acid sequence that is complimentary to a nucleic acid sequence of (a) or (b);

(c) a nucleic acid sequence that has substantial sequence homology to a nucleic acid sequence of (a), or (b);

(d) a nucleic acid sequence that is an analog of a nucleic acid sequence of (a), (b), or (c); or

(e) a nucleic acid sequence that hybridizes to a nucleic acid sequence of (a), (b), (c), or (d) under stringent hybridization conditions.

In a preferred embodiment the nucleic acid molecules of the invention encode teneurin c-terminal associated peptide that has anxiogenic activity.

The invention also encompasses antisense oligonucleotides complimentary to a nucleic acid sequence of the invention as well as expression vectors comprising a nucleic acid molecule of the invention and host cells transformed with the aforementioned expression vectors.

A further aspect of the invention relates to a method of identifying substances which can bind with a teneurin c-terminal associated peptide, comprising the steps of incubating a teneurin c-terminal associated peptide and a test substance, under conditions which allow for formation of a complex between the teneurin c-terminal associated peptide and the test substance, and assaying for complexes of the teneurin c-terminal associated peptide and the test substance, for free substance or for non complexed teneurin c-terminal associated peptide, wherein the presence of complexes indicates that the test substance is capable of binding a teneurin c-terminal associated peptide.

The invention also provides a method of identifying a compound that affects the regulation of neuronal growth comprising incubating a test compound with a teneurin c-terminal associated peptide or a nucleic acid

encoding a teneurin c-terminal associated peptide; and determining an amount of teneurin c-terminal associated peptide protein activity or expression and comparing with a control, wherein a change in the TCAP peptide activity or expression as compared to the control indicates that the test compound has an effect on the regulation of neuronal growth.

The invention also provides a method of inhibiting cell proliferation comprising administering to a cell, an effective amount of teneurin c-terminal associated peptide that inhibits cell proliferation. In a preferred embodiment, the inhibited cells are selected from the group consisting of neuronal or fibroblast cells.

Another aspect of the invention relates to a method of detecting a condition associated with the aberrant regulation of neuronal growth comprising assaying a sample for a nucleic acid molecule encoding a teneurin c-terminal associated peptide or a fragment thereof or a teneurin c-terminal associated peptide or a fragment thereof.

The invention also relates to a method of treating a condition associated with the aberrant regulation of neuronal growth, for instance cancer, comprising administering to a cell or animal in need thereof, an effective amount of teneurin c-terminal associated peptide or an agent that modulates teneurin c-terminal associated peptide expression and/or activity.

The teneurin-1 mRNA containing the TCAP-1 sequence is expressed in regions of the forebrain and limbic system regulating stress responses and anxiety. TCAP signals through a specific cAMP-dependent G-protein-coupled receptor to modify cell cycle and proliferation in immortalized neurons. Administration of synthetic TCAP-1 into the lateral ventricle or amygdala of rats normalized the acoustic startle response. These peptides, therefore, appear to be an integral part of the neural stress response and likely play a role in the aetiology of some psychiatric illnesses.

In another embodiment, the invention provides a method of modulating the stress response in an animal, preferably in a mammal, preferably a human, by administering to said animal an effective amount of TCAP, preferably TCAP-1 peptide, a nucleic acid molecule coding for said TCAP

peptide in a form that can express said peptide *in situ* or an antagonist or agonist of TCAP expression or activity, to modulate the stress response in said animal. In one embodiment the stress response is an anxiety response.

In another embodiment, the invention provides a method for normalizing the stress or anxiety response in an animal. In another embodiment, the invention provides a method for inducing an anxiogenic response in a low anxiety animal and for inducing an anxiolytic effect in a high anxiety animal.

In another embodiment, the invention provides a method modulating the stress response in an animal by modulating the effect of TCAP expression in an animal by administering to said animal a modulator of said TCAP expression or activity. In one embodiment said modulator is an inhibitor of TCAP expression and/or activity, in another embodiment, said modulator is an antagonist of TCAP expression or activity. In one embodiment said TCAP is TCAP-1.

In yet another embodiment, said invention provides a method of diagnosing an animal with high, normal or low stress response condition by administering to said animal a TCAP, such as TCAP-1 and monitoring whether it has an anxiolytic, anxiogenic or neutral effect on a stress response of the animal.

Other aspects of the invention relate to methods of inducing an anxiogenic response in a subject, methods of inhibiting damages caused by physiological stresses and methods of inhibiting cell death, each comprising administering to a subject an effective amount of teneurin c-terminal associated peptide for affecting the desired result.

Other features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will now be described in relation to the drawings in which:

Figure 1 shows a putative 3' exon of the rainbow trout Teneurin 3 gene [SEQ. ID. NO: 2] with an intron region [SEQ. ID. NO: 1] (1490 bp). The exon/intron border as established by sequence comparison with the human ten M1 gene (LocusLink ID# 10178) shown in the genome database. The intron placement was subsequently confirmed by PCR. The exon encodes the carboxy terminal 251 residues of the protein SEQ. ID. NO: 3. Cleavage signals are indicated in the bolded grey regions. The Terminal GKR motif usually signifies a post translation amidation signal. The teneurin-associated c-terminal peptide (TCAP) is shown by the sequence between amino acids 208 and 248 inclusive [SEQ. ID. NOS: 13 and 14].

Figure 2 shows the alignment of the amino acid sequences encoded by the terminal exon of the rainbow trout (*O. mykiss*) SEQ. ID.NO: 3, zebrafish (*R. danio*) SEQ. ID.NO: 12, mouse (*M. musculus*) SEQ. ID.NO: 6 and human (*H. sapiens*) SEQ. ID.NO: 10 genes. All possess an additional serine insertion in position 58. All show a high sequence similarity with about 94% between trout and zebrafish, 83% between rainbow trout and mouse, and 83% between rainbow trout and human. Within the TCAP portion itself, rainbow trout SEQ. ID. NO: 13 or 14 shares 90% sequence identity with zebrafish SEQ. ID. NO: 21 or 22, 90% sequence identity with mouse SEQ. ID. NO:53 or 54, and 88% with human SEQ. ID. NO. 85 or 86. The preTCAP sequences that include the amidation signal are SEQ. ID. NOS: 15 - 16 (Rainbow Trout), 23 - 24 (zebrafish), 55 - 56 (mouse) and 87 –88 (human).

Figure 3 shows the alignment of the amino acid sequences encoded by the terminal exon of the mouse teneurin 1, 2, 3 and 4) SEQ. ID. NOS: 4, 5, 6, 7 genes. The highest level of sequence similarity occurs among the sequences encoding the TCAP portion of the protein. TCAP-1 SEQ. ID. NO: 37 or 38 is 68% identical to TCAP-2 SEQ. ID. NO. 45 or 46, 76% identical to TCAP-3 SEQ. ID. NO. 53 or 54, and 85% identical to TCAP-4 SEQ. ID. NO. 61 or 62. TCAP-2 is 75% identical with TCAP-3, and 68% identical with

TCAP-4. TCAP-3 possesses 71 % identity with TCAP-4. Teneurin 3 possesses a dibasic cleavage site at the amino terminus of TCAP-3 whereas 1, 2 and 4 all possess monobasic sites suggesting that the cleaved peptide is 40 residues in TCAP-3 but 41 residues in TCAP-1, 2 and 4. However, in one embodiment, both the 41 and 40 amino acid residue TCAP has activity.

Figure 4 shows the alignment of amino acid sequences encoded by the last exon of the human Teneurin 1, 2, 3 and 4 proteins SEQ. ID. NOS: 8, 9, 10, 11. Like the mouse sequence, the highest degree of sequence similarity occurs in the TCAP portion of the exon. TCAP-3 possesses a dibasic leaved signal whereas the others possess a monobasic site. The greatest variable region occurs with the first 70- 80 residues of the exon. Within the TCAP portion itself, TCAP-1 SEQ. ID. NO: 69 or 70 shares 73% identity with TCAP-2 SEQ. ID. NO: 77 or 78, 83% identity with TCAP-3 SEQ. ID. NO: 85 or 86 and 88% identity with TCAP-4 SEQ. ID. NO. 93 or 94. TCAP-2 has 76% identity with TCAP-3 and 71% identity with TCAP-4. TCAP-3 has 76% identity with TCAP-4.

Figure 5 shows the nucleotide coding sequence of the preTCAP sequences for Human (SEQ. ID. NOS: 76, 84, 92, and 100) and Mouse (SEQ. ID. NOS. 44, 52, 60 and 68) preTCAP-1 to 4, Zebrafish preTCAP-3 and 4 (SEQ. ID. NOS: 28 and 36), and Rainbow Trout preTCAP-3 (SEQ. ID. NO. 20) with stop codon. The coding region of the corresponding mature TCAP peptides would lack the terminal amidation and stop codon coding sequence (e.g. the last 12 nucleotide bases shown for each sequence). The sequences shown code for the 44 amino acid residue preTCAP sequence with stop codon. However, the 43 amino acid TCAP coding sequnce is identical except with the first three nucleotides absent.

Figure 6A is a schematic representation of the functional domains within the Teneurin protein. Figure 6B is a schematic view of the exons on human teneurin 1 and an exploded view of the location of the C-terminal exon and location of TCAP thereon. A conserved prohormone convertase-like cleavage motif is shown as grey boxes. It illustrates the structure of Teneurin

C-terminal Associated Peptides and their location on the teneurin protein and gene.

Figure 7A shows the alignment of the human, mouse, rat, chicken, rainbow trout, zebrafish and drosopholia TCAP sequences SEQ. ID.NOS: 69, 78, 85, 94, 37, 46, 53, 66, 78, 101, 136, 13, 21, 30 and 103 and 7B shows the alignment of the TCAP sequences from mammals birds insects and nematodes Fig. 7B SEQ. ID. NOS: 37, 101 (without the Q at the N-terminus), 69, 61, 93, 53, 85, 13, 21, 77, 30, and 103. In figure 7B, non homologous amino acid substitutions are shaded in light grey. Homologous residues are shaded in dark grey.

Figure 8 shows the alignment of the amino acid sequences of the human CRF family SEQ. ID. NOS: 104 – 107 with those of the human TCAP family SEQ. ID.NOS: 70, 78, 85, 94. Although overall sequence identity is only about 20-25 %, many of the other substitutions reflect potential single base codon changes such as proline to serine, leucine or threonine, or conservative amino acid substitutions such as leucine to valine or isoleucine, aspartic acid to glutamic acid and asparagines to glutamine.

Figure 9 is a comparison of the sequence identity among CRF family members to that of the identity among TCAP members. The TCAP family members show a much greater sequence identity of 68% compared to the CRF family members of 34% between CRF and U3 and U2, 43% between CRF and urocortin, and 21% between urocortin 1 and 3.

Figure 10 shows a secondary structure prediction of TCAP (Rainbow Trout TCAP-3) and comparison with CRF-like peptides. Figure 10 A is a Grantham Polarity Prediction and Figure 10B is a Kyte-Doolittle Hydrophobicity Prediction. TCAP shows a highly similar polarity profile, but appears to possess higher levels of total hydrophobicity in the amino terminus.

Figure 11 shows the alignment of amino acid sequences of representations of TCAP peptides with the insect diuretic peptides and CRF superfamily SEQ. ID. NOS: 13, 22, 104, 107-110. The entire superfamily can

be divided into three general regions encompassing an amino terminal portion, a midsection and a carboxy terminal portion. All peptides can be aligned by the presence of conserved motifs within each of the separate sections

Figure 12 illustrates expression of Teneurins in mouse brain and cell lines NLT, Gn11, and Nero2a . PCR-amplified products corresponding to Teneurin 1 to 4 were found in whole brain and cell lines. TenM1,2 and 4 were found in whole brain and in the immortalized GnRH-expressing neuronal line, Gn11. Only Teneurin 2 and 4 were found in another GnRH-expressing cell, NLT, however, all four forms were found in the Neuro2a neuroblastoma cell line. The bands on top indicate positive signals for the Teneurin transcripts. The bands at the bottom show a positive signal for glyceraldehydes-3-phosphate dehydrogenase (GAPDH) to indicate the viability of the RNA. A 100-bp DNA ladder is shown at the left of all PCR gels.

Figure 13 is a bar graph illustrating the inhibition of cell proliferation in Gn11 neuronal cells by 10^{-6} M TCAP (Rainbow Trout TCAP-3) at 48 hours (Figure 13 A) and at 72 hours (Figure 13B).

Figure 14 is a bar graph illustrating the inhibition of cell proliferation in TGR1 (wildtype) fibroblast cells.

Figure 15 is a bar graph illustrating the inhibition of cell proliferation in HO16 (c-myc constitutively expressed cells) (14B) by 10^{-6} M TCAP (Rainbow Trout TCAP-3) at 48 hours).

Figures 16A and 16B are bar graphs illustrating the inhibition of cAMP(16A) and cGMP (16B) accumulation in Gn11 cells by rtTCAP-3(Rainbow Trout TCAP-3). A. 10^{-6} M TCAP induced a significant ($p<0.01$) decrease in cAMP concentrations relative to the vehicle-treated cells. Replications: vehicle, n=10; urocortin, n=8; TCAP, n=11. B. 10^{-6} M TCAP induced a significant ($p<0.01$) decrease in cGMP accumulation in Gn11 cells. The same concentration of rat urocortin also induced a significant ($p<0.05$) decrease in cGMP concentrations. Three replications were used for each of the treatment groups. Significance was assessed using a one-way analysis of

variance with a Dunnett's post-hoc test. An a priori level of significance was established at p=0.05. The original data was transformed to show percent concentration relative to the vehicle-treated cells.

Figure 17 A-D illustrates TCAP(Rainbow Trout TCAP-3) cAMP regulation in Gn11 cells. 17A illustrates cAMP levels in Gn11 cells treated with 10^{-8} M TCAP or urocortin over 30 minutes. 17B illustrates cAMP levels in Gn11 cells in the presence of 10^{-4} M 3-isobutyl-1 methyl xanthine (IBMX), a phosphodiesterase inhibitor used to boost cAMP induced by treatment of 10^{-8} MTCAP or urocortin. 17C is a bar graph illustrating cAMP accumulation over 30 minutes in Gn11 cells by administration of various concentrations of TCAP or Urocortin in the presence of IBMX. 17D is a bar graph illustrating inhibition of 10^{-6} M forskolin- stimulated cAMP by 10^{-8} MTCAP or urocortin.

Figures 18A and 18B are linear graphs illustrating the effect of TCAP (Rainbow Trout TCAP-3) on the administration of self reward behaviour. The behaviour was indicated by number of bar presses per 30 seconds over a range of pleasurable stimulation (25 – 100Hz). Figure 18A: Baseline, TCAP peptide (1.0µl of 0.001mg/ml, left), post-injection (approx. 90 min.), 850uA. Figure 18B: Baseline, TCAP peptide ((1.0µl of 0.001mg/ml, right), postinjection (approx. 60 min.), 550uA. 100 nM TCAP induced a significant decrease in the rats desire to self-administer reward by neural impulse.

Figure 19 A schematic cellular model for TCAP regulation. A. A stressor in the form of a physiological condition such as low oxygen or pH changes, or an anxiogenic ligand triggers metabolic activation of the cell. B. This causes an upregulation of the Teneurin protein and its cleaving enzyme. C. The enzyme liberates TCAP from Teneurin where it acts in an autocrine and paracrine manner to inhibit cAMP and cGMP production via a G protein coupled receptor.

Figure 20 illustrates the distribution of TCAP-1 mRNA in rat brain nuclei as explained in Example 9.

Figure 21 are bar graphs illustrating the chronic human TCAP-1 response in rats that were (A) vehicle treated ICV injected, (B)TCAP-1 ICV injected as described in Example 10 herein.

Figure 22 are graphs illustrating the mean baseline startle response of all animals in Example 10. Figure 22A is the average startle response at day 1 after TCAP injection and Figure 22B is the average startle at the end of the chronic TCAP study, Figure 22C is the average startle response following TCAP-1.

Figure 23 is the interaction bar plot for treatment with TCAP-1 at various doses for both high and low anxiety response animals as discussed in Example 11 herein.

Figure 24 is the plot of the effect of TCAP-1 amygdala –injected on the startle response of rats as discussed in Example 11 herein.

Figure 25 illustrates activity of TCAP on immortalized neurons. (A) cAMP accumulation in Gn11 cells. 1nM TCAP increased cAMP ($p<0.05$) whereas 100 nM TCAP decreased ($p<0.05$) cAMP. An intermediate concentration (10nM) was without effect. (B) Action of CRF-R1 antagonist on cAMP accumulation. A 1 nM mouse TCAP-1, or mouse urocortin increased cAMP accumulation in Gn11 cells. The CRF R1 receptor antagonist PD171729 abolished the action of urocortin on these cells ($p<0.01$) but had no effect on TCAP-mediated cAMP accumulation. (C) Protein assays. Concentrations of 1 to 100 nM TCAP stimulated protein synthesis in Gn11 cells. (D) MTT Assay. 1 nM of mouse TCAP-1 increased MTT activity ($p<0.05$) in Gn11 cells after 48 hours. In contrast, 100 nM of mouse TCAP-1 decreased ($p<0.05$) MTT activity over the same time period. E. DNA Content Analysis. mouse TCAP-1 reduced the incidence of G1 phase at the lowest concentration of 1 nM, however, increased the number of cells in G1 phase at the highest dose of 100 nM. The level of significance was determined using a one-way ANOVA for A, B and E, and a two-way ANOVA for C and D.

Figure 26 illustrates the functional cAMP response of murine hypothalamic immortalized cell lines to TCAP(rainbow trout TCAP-3) peptide stimulation.

DETAILED DESCRIPTION OF THE INVENTION

The inventors have identified a novel peptide sequence which exists as part of a larger protein previously identified as the Ten M proteins or Teneurins. The novel peptides are referred to as teneurin C terminal peptides or TCAP. The genomes or gene transcripts of several vertebrate and invertebrate species were screened by homologous probe hybridization or by PCR. Sequence data from genome sequencing programs allowed the identification of a complement of four paralogous peptides from this family in humans and mice, two paralogues in zebrafish, one in rainbow trout and Drosophila (SEQ ID NO:103). The synthetic TCAP peptide has neuronal comunciation activity and has been shown to be a modulator of the stress response and anxiety in an animal. TCAp also modulates cell proliferation. In one embodiment , it can inhibit cell proliferation. In another emboimdnent, TCAPis a potent anxiogenic peptide in rats and highly effective at inhibiting neuronal proliferation in unstressed cells and protecting cells from physiological stresses. As such TCAP and/or modulators of TCAP can be used in the treatment of cancer and neuropathological conditions, including those related to neuronal communication, and.or cell proliferation, for instance, cancer, stress anxiety, food-related disorders, such as anorexia and/or obesity.

The TCAP sequence encodes a cleavable peptide 40 amino acids long flanked by PC7 -like cleavage motifs on the amino terminus and an amidation motif on the carboxy terminus. Depending on the cleavage of the PC7-like cleavage site at the N-terminus, the resulting mature TCAP peptide is 40-41 amino acids in length. The TCAP sequence with the carboxy terminus amidation motif is herein referred to as preTCAP. Orthologues in humans, mice, zebrafish and Drosophila as well as three additional paralogous sequences have been identified. A synthetic version of the rainbow trout peptide significantly increases the startle reflex and decreases self-administered brain stimulation in rats. These findings are consistent with the actions of peptides known to induce anxiety in mammals and humans. The peptide is also potent at inhibiting the proliferation of unstressed neuronal and fibroblast cell cultures and inhibiting cell death in these cultures subjected to

high pH stress. These findings indicate that TCAP plays a role in the developing and adult brain to modulate and protect neuronal growth and metabolism and therefore be implicated in a number of pathologies including schizophrenia, Parkinson's disease and other mental conditions. In the adult brain the peptide may act to modulate the actions of anxiogenic stimuli and could play a role in depression, anorexia nervosa and other affective disorders.

The term "isolated" as used herein means "altered by the hand of man" from the natural state. If a composition or substance occurs in nature, the isolated form has been changed or removed from its original environment, or both. For example, a polynucleotide or a polypeptide naturally present in a living animal is not "isolated," but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated," as the term is employed herein. Thus, a polypeptide or polynucleotide produced and/or contained within a recombinant host cell is considered isolated for purposes of the present invention. Also intended as an "isolated polypeptide" or an "isolated polynucleotide" are polypeptides or polynucleotides that have been purified, partially or substantially, from a recombinant host cell or from a native source. For example, a recombinantly produced version of TCAP peptides and derivatives thereof can be substantially purified by methods known in the art, such as the one-step method described in Smith and Johnson, Gene 67:31-40 (1988).

Nucleic Acid Molecules of the Invention

The present invention provides an isolated nucleic acid molecule consisting of a sequence encoding a teneurin c-terminal associated peptide This peptide is generally referred to as "TCAP" herein. The present invention also provides an isolated nucleic acid molecule encoding a TCAP peptide with a carboxy terminus amidation motif, said peptide herein referred to as "preTCAP".

Isolated nucleic acids substantially free of cellular material or culture medium when produced by recombinant DNA techniques, or chemical

precursors, or other chemicals when chemically synthesized are included in this invention.

In a preferred embodiment, the invention provides an isolated nucleic acid sequence comprising or consisting of:

(a) a nucleic acid sequence as shown in SEQ.ID.NOS.: 18-20, 25-28, 33-36, 41-44, 49-52, 57-60, 65-68, 73-76, 81-84, 89-92, 97-100 or that wherein T can also be U or that encodes a peptide having an amino acid sequence selected from the group consisting of : SEQ. ID. NOS: 13, 14, 21, 22, 29, 30, 37, 38, 45, 46, 53, 54, 61, 62, 69, 70, 77, 78, 85, 86, 93, 94, 101, 103 or that further has an amidation signal sequence (preferably GKR or GRR), at the carboxy terminus of said peptides, such as 15, 16, 23, 24, 31, 32, 39, 40, 47, 48, 55, 56, 63, 64, 71, 72, 79, 80, 97, 88, 95, 96;

(b) a nucleic acid sequence that is complimentary to a nucleic acid sequence of (a);

(c) a nucleic acid sequence that has substantial sequence homology to a nucleic acid sequence of (a) or (b);

(d) a nucleic acid sequence that is an analog of a nucleic acid sequence of (a), (b) or (c); or

(e) a nucleic acid sequence that hybridizes to a nucleic acid sequence of (a), (b), (c) or (d) under stringent hybridization conditions.

(f) a nucleice acid sequence of (a) –(e) where T is U.

The term "sequence that has substantial sequence homology" means those nucleic acid sequences which have slight or inconsequential sequence variations from the sequences in (a) or (b), i.e., the sequences function in substantially the same manner. The variations may be attributable to local mutations or structural modifications. Nucleic acid sequences having substantial homology include nucleic acid sequences having at least 65%, more preferably at least 85%, and most preferably 90-95% identity with the nucleic acid sequences as listed in (a) above. The term "sequence that hybridizes" means a nucleic acid sequence that can hybridize to a sequence of (a), (b), (c) or (d) under stringent hybridization conditions. Appropriate "stringent hybridization conditions" which promote DNA hybridization are

known to those skilled in the art, or may be found in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. For example, the following may be employed: 6.0 x sodium chloride/sodium citrate (SSC) at about 45°C, followed by a wash of 2.0 x SSC at 50°C; 0.2 x SSC at 50°C to 65°C; or 2.0 x SSC at 44°C to 50°C. The stringency may be selected based on the conditions used in the wash step. For example, the salt concentration in the wash step can be selected from a high stringency of about 0.2 x SSC at 50°C. In addition, the temperature in the wash step can be at high stringency conditions, at about 65°C.

The term "nucleic acid" is intended to include DNA and RNA and can be either double stranded or single stranded.

The term "a nucleic acid sequence which is an analog" means a nucleic acid sequence which has been modified as compared to the sequence of (a), (b) or (c) wherein the modification does not alter the utility of the sequence as described herein. The modified sequence or analog may have improved properties over the sequence shown in (a), (b) or (c). One example of a modification to prepare an analog is to replace one of the naturally occurring bases (i.e. adenine, guanine, cytosine or thymidine) of the sequence with a modified base such as such as xanthine, hypoxanthine, 2-aminoadenine, 6-methyl, 2-propyl and other alkyl adenines, 5-halo uracil, 5-halo cytosine, 6-aza uracil, 6-aza cytosine and 6-aza thymine, pseudo uracil, 4-thiouracil, 8-halo adenine, 8-aminoadenine, 8-thiol adenine, 8-thiolalkyl adenines, 8-hydroxyl adenine and other 8-substituted adenines, 8-halo guanines, 8 amino guanine, 8-thiol guanine, 8-thiolalkyl guanines, 8-hydroxyl guanine and other 8-substituted guanines, other aza and deaza uracils, thymidines, cytosines, adenines, or guanines, 5-trifluoromethyl uracil and 5-trifluoro cytosine.

Another example of a modification is to include modified phosphorous or oxygen heteroatoms in the phosphate backbone, short chain alkyl or cycloalkyl intersugar linkages or short chain heteroatomic or heterocyclic intersugar linkages in the nucleic acid molecule listed in (a) to (e) above. For

example, the nucleic acid sequences may contain phosphorothioates, phosphotriesters, methyl phosphonates, and phosphorodithioates.

A further example of an analog of a nucleic acid molecule of the invention is a peptide nucleic acid (PNA) wherein the deoxyribose (or ribose) phosphate backbone in the DNA (or RNA), is replaced with a polyamide backbone which is similar to that found in peptides (P.E. Nielsen, et al Science 1991, 254, 1497). PNA analogs have been shown to be resistant to degradation by enzymes and to have extended lives *in vivo* and *in vitro*. PNAs also bind stronger to a complimentary DNA sequence due to the lack of charge repulsion between the PNA strand and the DNA strand. Other nucleic acid analogs may contain nucleotides containing polymer backbones, cyclic backbones, or acyclic backbones. For example, the nucleotides may have morpholino backbone structures (U.S. Pat. No. 5,034,506). The analogs may also contain groups such as reporter groups, a group for improving the pharmacokinetic or pharmacodynamic properties of nucleic acid sequence.

Isolated and purified nucleic acid molecules having sequences which differ from the nucleic acid sequence of the invention due to degeneracy in the genetic code are also within the scope of the invention. Such nucleic acids encode functionally equivalent peptides but differ in sequence from the above mentioned sequences due to degeneracy in the genetic code.

An isolated nucleic acid molecule of the invention which consists of DNA can be isolated by preparing a labeled nucleic acid probe based on all or part of the nucleic acid sequences of the invention and using this labeled nucleic acid probe to screen an appropriate DNA library (e.g. a cDNA or genomic DNA library). For example, a genomic library isolated can be used to isolate a DNA encoding a novel peptide of the invention by screening the library with the labeled probe using standard techniques. Nucleic acids isolated by screening of a cDNA or genomic DNA library can be sequenced by standard techniques.

An isolated nucleic acid molecule of the invention which is DNA can also be isolated by selectively amplifying a nucleic acid encoding a novel peptide of the invention using the polymerase chain reaction (PCR) methods

and cDNA or genomic DNA. It is possible to design synthetic oligonucleotide primers from the nucleic acid sequence of the invention for use in PCR. A nucleic acid can be amplified from cDNA or genomic DNA using these oligonucleotide primers and standard PCR amplification techniques. The nucleic acid so amplified can be cloned into an appropriate vector and characterized by DNA sequence analysis. It will be appreciated that cDNA may be prepared from mRNA, by isolating total cellular mRNA by a variety of techniques, for example, by using the guanidinium-thiocyanate extraction procedure of Chirgwin et al., Biochemistry, 18, 5294-5299 (1979). cDNA is then synthesized from the mRNA using reverse transcriptase (for example, Moloney MLV reverse transcriptase available from Invitrogen, Carlsbad, CA, or AMV reverse transcriptase available from Seikagaku America, Inc., St. Petersburg, FL).

An isolated nucleic acid molecule of the invention which is RNA can be isolated by cloning a cDNA encoding a novel peptide of the invention into an appropriate vector which allows for transcription of the cDNA to produce an RNA molecule which encodes a protein of the invention. For example, a cDNA can be cloned downstream of a bacteriophage promoter, (e.g., a T7 promoter) in a vector, cDNA can be transcribed *in vitro* with T7 polymerase, and the resultant RNA can be isolated by standard techniques.

A nucleic acid molecule of the invention may also be chemically synthesized using standard techniques. Various methods of chemically synthesizing polydeoxynucleotides are known, including solid-phase synthesis which, like peptide synthesis, has been fully automated in commercially available DNA synthesizers (See e.g., Itakura et al. U.S. Patent No. 4,598,049; Caruthers et al. U.S. Patent No. 4,458,066; and Itakura U.S. Patent Nos. 4,401,796 and 4,373,071).

Determination of whether a particular nucleic acid molecule encodes a novel peptide of the invention may be accomplished by expressing the cDNA in an appropriate host cell by standard techniques, and testing the activity of the peptide using the methods as described herein. A cDNA having the activity of a novel peptide of the invention so isolated can be sequenced by

standard techniques, such as dideoxynucleotide chain termination or Maxam-Gilbert chemical sequencing, to determine the nucleic acid sequence and the predicted amino acid sequence of the encoded peptide.

The initiation codon and untranslated sequences of nucleic acid molecules of the invention may be determined using currently available computer software designed for the purpose, such as PC/Gene (IntelliGenetics Inc., Calif.). Regulatory elements can be identified using conventional techniques. The function of the elements can be confirmed by using these elements to express a reporter gene which is operatively linked to the elements. These constructs may be introduced into cultured cells using standard procedures. In addition to identifying regulatory elements in DNA, such constructs may also be used to identify proteins interacting with the elements, using techniques known in the art.

The sequence of a nucleic acid molecule of the invention may be inverted relative to its normal presentation for transcription to produce an antisense nucleic acid molecule which are more fully described herein. In particular, the nucleic acid sequences contained in the nucleic acid molecules of the invention or a fragment thereof, may be inverted relative to its normal presentation for transcription to produce antisense nucleic acid molecules.

The invention also provides nucleic acids encoding fusion proteins comprising a novel protein of the invention and a selected protein, or a selectable marker protein (see below).

Also provided are portions of the nucleic acid sequence encoding fragments, functional domains or antigenic determinants of the TCAP peptide. The present invention also provides for the use of portions of the sequence as probes and PCR primers for TCAP as well as for determining functional aspects of the sequence.

One of ordinary skill in the art is now enabled to identify and isolate TCAP encoding nucleic acids or cDNAs that are allelic variants of the disclosed sequences, using standard hybridization screening or PCR techniques.

II. Novel Proteins of the Invention

The invention further broadly contemplates an isolated TCAP peptide. The term "TCAP peptide" as used herein includes all homologs, analogs, fragments or derivatives of the TCAP peptide.

The term "analog" in reference to peptides includes any peptide having an amino acid residue sequence substantially identical to the human or mouse TCAP sequence specifically shown herein in which one or more residues have been conservatively substituted with a functionally similar residue and which displays the ability to mimic TCAP as described herein. Examples of conservative substitutions include the substitution of one non-polar (hydrophobic) residue such as alanine, isoleucine, valine, leucine or methionine for another, the substitution of one polar (hydrophilic) residue for another such as between arginine and lysine, between glutamine and asparagine, between glycine and serine, the substitution of one basic residue such as lysine, arginine or histidine for another, or the substitution of one acidic residue, such as aspartic acid or glutamic acid for another. The phrase "conservative substitution" also includes the use of a chemically derivatized residue in place of a non-derivatized residue provided that such polypeptide displays the requisite activity.

The term "derivative" reference to peptides refers to a peptide having one or more residues chemically derivatized by reaction of a functional side group. Such derivatized molecules include for example, those molecules in which free amino groups have been derivatized to form amine hydrochlorides, p-toluene sulfonyl groups, carbobenzoxy groups, t-butyloxycarbonyl groups, chloroacetyl groups or formyl groups. Free carboxyl groups may be derivatized to form salts, methyl and ethyl esters or other types of esters or hydrazides. Free hydroxyl groups may be derivatized to form O-acyl or O-alkyl derivatives. The imidazole nitrogen of histidine may be derivatized to form N-im-benzylhistidine. Also included as derivatives are those peptides which contain one or more naturally occurring amino acid derivatives of the twenty standard amino acids. For examples: 4-hydroxyproline may be substituted for proline; 5-hydroxylysine may be substituted for lysine; 3-methylhistidine may be substituted for histidine; homoserine may be

substituted for serine; and ornithine may be substituted for lysine. Polypeptides of the present invention also include any polypeptide having one or more additions and/or deletions or residues relative to the sequence of a polypeptide whose sequence is shown herein, so long as the requisite activity is maintained.

In one embodiment, the isolated TCAP peptide consists of 38-41 amino acid residues of the carboxy terminus of a teneurin-like protein with or without an amidation signal at the carboxy terminus. In one embodiment, the amidation signal consists of the amino acid sequence GKR or GRR (preTCAP). In another embodiment, the TCAP peptide comprises sequences substantially identity to the above-noted peptides or comprising an obvious chemical equivalents thereof. It also includes peptides sequence +/- amino acids at the amino and/or carboxy terminus of the above-noted TCAP peptide sequences. In yet another embodiment, the invention includes fusion proteins, comprising the TCAP peptide, labeled TCAP peptides, analogs, homologs and variants thereof.

In one embodiment, the TCAP peptide is a rainbow trout, zebrafish, human, mouse, *G. gallus* or *D. melanogaster* TCAP. In another embodiment, the TCAP peptides have the sequence selected from the group consisting of: SEQ. ID. NOS: 13, 14, 21, 22, 29, 30, 37, 38, 45, 46, 53, 54, 61, 62, 69, 70, 77, 78, 85, 86, 93, 94, 101, 103 or that further has an amidation signal sequence (preferably GKR or GRR), at the carboxy terminus of said peptides, such as 15, 16, 23, 24, 31, 32, 39, 40, 47, 48, 55, 56, 63, 64, 71, 72, 79, 80, 97, 88, 95, 96;

In another embodiment, the peptide of the invention is a TCAP-3 and has the following amino acid sequence motif:

QLLSXaa$_1$Xaa$_2$KVXaa$_3$GYDGYYVLSXaa$_4$EQYPELADSANNXaa$_5$QFL RQSEI SEQ. ID. NO:135

Where Xaa$_1$ is G, S, or A; Xaa$_2$ is G or R; Xaa$_3$ is L or Q; Xaa$_4$ and Xaa$_5$ are independently V or I. In one embodiment, the TCAP-3 is a human or mouse TCAP- 3. In another embodiment, the TCAP- 3 has SEQ. ID. NO: 13, 21, 53 or 85.

Within the context of the present invention, a peptide of the invention may include various structural forms of the primary peptide which retain biological activity. For example, a peptide of the invention may be in the form of acidic or basic salts or in neutral form. In addition, individual amino acid residues may be modified by oxidation or reduction.

In addition to the full-length amino acid sequence, the peptide of the present invention may also include truncations, analogs and homologs of the peptide and truncations thereof as described herein. Truncated peptides or fragments may comprise peptides of at least 5, preferably 10 and more preferably 15 amino acid residues of the sequence listed above.

The invention further provides polypeptides comprising at least one functional domain or at least one antigenic determinant of a TCAP peptide.

Analogs of the protein of the invention and/or truncations thereof as described herein, may include, but are not limited to an amino acid sequence containing one or more amino acid substitutions, insertions, deletions and/or mutations. Amino acid substitutions may be of a conserved or non-conserved nature. Conserved amino acid substitutions involve replacing one or more amino acids of the peptides of the invention with amino acids of similar charge, size, and/or hydrophobicity characteristics. When only conserved substitutions are made the resulting analog should be functionally equivalent. Non-conserved substitutions involve replacing one or more amino acids of the amino acid sequence with one or more amino acids which possess dissimilar charge, size, and/or hydrophobicity characteristics.

One or more amino acid insertions may be introduced into the amino acid sequences of the invention. Amino acid insertions may consist of single amino acid residues or sequential amino acids ranging from 2 to 15 amino acids in length. For example, amino acid insertions may be used to destroy target sequences so that the peptide is no longer active. This procedure may be used *in vivo* to inhibit the activity of the peptide of the invention.

Deletions may consist of the removal of one or more amino acids, or discrete portions from the amino acid sequence of the TCAP peptide. The deleted amino acids may or may not be contiguous.

Analogs of a protein of the invention may be prepared by introducing mutations in the nucleotide sequence encoding the peptide. Mutations may be introduced at particular loci by synthesizing oligonucleotides containing a mutant sequence, flanked by restriction sites enabling ligation to fragments of the native sequence. Following ligation, the resulting reconstructed sequence encodes an analog having the desired amino acid insertion, substitution, or deletion.

Alternatively, oligonucleotide-directed site-specific mutagenesis procedures may be employed to provide an altered gene having particular codons altered according to the substitution, deletion, or insertion required. Deletion or truncation of a peptide of the invention may also be constructed by utilizing convenient restriction endonuclease sites adjacent to the desired deletion. Subsequent to restriction, overhangs may be filled in, and the DNA religated. Exemplary methods of making the alterations set forth above are disclosed by Sambrook et al (Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, 1989).

The peptides of the invention also include homologs of the amino acid sequence of the TCAP peptide, mutated TCAP peptides and/or truncations thereof as described herein. Such homologs are proteins whose amino acid sequences are comprised of amino acid sequences that hybridize under stringent hybridization conditions (see discussion of stringent hybridization conditions herein) with a probe used to obtain a peptide of the invention. Homologs of a peptide of the invention will have the same regions which are characteristic of the protein.

A homologous peptide includes a peptide with an amino acid sequence having at least 70%, preferably 80-95% identity with the amino acid sequence of the TCAP peptide.

The invention also contemplates isoforms of the peptides of the invention. An isoform contains the same number and kinds of amino acids as a peptide of the invention, but the isoform has a different molecular structure.The isoforms contemplated by the present invention are those having the same properties as a peptide of the invention as described herein.

The proteins of the invention (including e.g., truncations, analogs, etc.) may be prepared using recombinant DNA methods. Accordingly, nucleic acid molecules of the present invention having a sequence that encodes a peptide of the invention may be incorporated according to procedures known in the art into an appropriate expression vector that ensures good expression of the peptide. Possible expression vectors include but are not limited to cosmids, plasmids, or modified viruses (e.g., replication defective retroviruses, adenoviruses and adeno-associated viruses), so long as the vector is compatible with the host cell used. The expression "vectors suitable for transformation of a host cell", means that the expression vectors contain a nucleic acid molecule of the invention and regulatory sequences, selected on the basis of the host cells to be used for expression, which are operatively linked to the nucleic acid molecule. "Operatively linked" is intended to mean that the nucleic acid is linked to regulatory sequences in a manner that allows expression of the nucleic acid.

The invention therefore contemplates a recombinant expression vector of the invention containing a nucleic acid molecule of the invention, or a fragment thereof, and the necessary regulatory sequences for the transcription and translation of the inserted peptide-sequence. Suitable regulatory sequences may be derived from a variety of sources, including bacterial, fungal, or viral genes (For example, see the regulatory sequences described in Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990). Selection of appropriate regulatory sequences is dependent on the host cell chosen, and may be readily accomplished by one of ordinary skill in the art. Examples of such regulatory sequences include: a transcriptional promoter and enhancer or RNA polymerase binding sequence, a ribosomal binding sequence, including a translation initiation signal. Additionally, depending on the host cell chosen and the vector employed, other sequences, such as an origin of replication, additional DNA restriction sites, enhancers, and sequences conferring inducibility of transcription may be incorporated into the expression vector. It

will also be appreciated that the necessary regulatory sequences may be supplied by the native peptide and/or its flanking regions.

The invention further provides a recombinant expression vector comprising a DNA nucleic acid molecule of the invention cloned into the expression vector in an antisense orientation. That is, the DNA molecule is operatively linked to a regulatory sequence in a manner that allows for expression, by transcription of the DNA molecule, of an RNA molecule which is antisense to a nucleotide sequence of the invention. Regulatory sequences operatively linked to the antisense nucleic acid can be chosen which direct the continuous expression of the antisense RNA molecule.

The recombinant expression vectors of the invention may also contain a selectable marker gene that facilitates the selection of host cells transformed or transfected with a recombinant molecule of the invention. Examples of selectable marker genes are genes encoding a protein such as G418 and hygromycin which confer resistance to certain drugs, β-galactosidase, chloramphenicol acetyltransferase, or firefly luciferase. Transcription of the selectable marker gene is monitored by changes in the concentration of the selectable marker protein such as β-galactosidase, chloramphenicol acetyltransferase, or firefly luciferase. If the selectable marker gene encodes a protein conferring antibiotic resistance such as neomycin resistance transformant cells can be selected with G418. Cells that have incorporated the selectable marker gene will survive, while the other cells die. This makes it possible to visualize and assay for expression of recombinant expression vectors of the invention and in particular to determine the effect of a mutation on expression and phenotype. It will be appreciated that selectable markers can be introduced on a separate vector from the nucleic acid of interest.

Recombinant expression vectors can be introduced into host cells to produce a transformed host cell. Accordingly, the invention includes a host cell comprising a recombinant expression vector of the invention. The term "transformed host cell" is intended to include prokaryotic and eukaryotic cells which have been transformed or transfected with a recombinant expression

vector of the invention. The terms "transformed with", "transfected with", "transformation" and "transfection" are intended to encompass introduction of nucleic acid (e.g. a vector) into a cell by one of many possible techniques known in the art. Prokaryotic cells can be transformed with nucleic acid by, for example, electroporation or calcium-chloride mediated transformation. Nucleic acid can be introduced into mammalian cells via conventional techniques such as calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofectin, electroporation or microinjection. Suitable methods for transforming and transfecting host cells can be found in Sambrook et al. (Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory press (1989)), and other such laboratory textbooks.

Suitable host cells include a wide variety of prokaryotic and eukaryotic host cells. For example, the peptides of the invention may be expressed in bacterial cells such as *E. coli*, *Pseudomonas*, *Bacillus subtillus*, insect cells (using baculovirus), yeast cells or mammalian cells. Other suitable host cells can be found in Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1991).

As an example, to produce TCAP peptides recombinantly, for example, *E. coli* can be used using the T7 RNA polymerase/promoter system using two plasmids or by labeling of plasmid-encoded proteins, or by expression by infection with M13 Phage mGPI-2. *E. coli* vectors can also be used with Phage lamba regulatory sequences, by fusion protein vectors (e.g. lacZ and trpE), by maltose-binding protein fusions, and by glutathione-S-transferase fusion proteins.

Alternatively, a TCAP peptide can be expressed in insect cells using baculoviral vectors, or in mammalian cells using vaccinia virus. For expression in mammalian cells, the cDNA sequence may be ligated to heterologous promoters and introduced into cells, such as COS cells to achieve transient or long-term expression. The stable integration of the chimeric gene construct may be maintained in mammalian cells by biochemical selection, such as neomycin and mycophoenolic acid.

The TCAP DNA sequence can be altered using procedures such as restriction enzyme digestion, fill-in with DNA polymerase, deletion by exonuclease, extension by terminal deoxynucleotide transferase, ligation of synthetic or cloned DNA sequences, site-directed sequence alteration with the use of specific oligonucleotides together with PCR.

The cDNA sequence or portions thereof, or a mini gene consisting of a cDNA with an intron and its own promoter, is introduced into eukaryotic expression vectors by conventional techniques. These vectors permit the transcription of the cDNA in eukaryotic cells by providing regulatory sequences that initiate and enhance the transcription of the cDNA and ensure its proper splicing and polyadenylation. The endogenous TCAP gene promoter can also be used. Different promoters within vectors have different activities which alters the level of expression of the cDNA. In addition, certain promoters can also modulate function such as the glucocorticoid-responsive promoter from the mouse mammary tumor virus.

Some of the vectors listed contain selectable markers or neo bacterial genes that permit isolation of cells by chemical selection. Stable long-term vectors can be maintained in cells as episomal, freely replicating entities by using regulatory elements of viruses. Cell lines can also be produced which have integrated the vector into the genomic DNA. In this manner, the gene product is produced on a continuous basis.

Vectors are introduced into recipient cells by various methods including calcium phosphate, strontium phosphate, electroporation, lipofection, DEAE dextran, microinjection, or by protoplast fusion. Alternatively, the cDNA can be introduced by infection using viral vectors.

TCAP peptides may also be isolated from cells or tissues, including mammalian cells or tissues, in which the peptide is normally expressed.

The protein may be purified by conventional purification methods known to those in the art, such as chromatography methods, high performance liquid chromatography methods or precipitation.

For example, an anti-TCAP antibody (as described below) may be used to isolate a TCAP peptide, which is then purified by standard methods.

The peptides of the invention may also be prepared by chemical synthesis using techniques well known in the chemistry of proteins such as solid phase synthesis (Merrifield, 1964, J. Am. Chem. Assoc. 85:2149-2154) or synthesis in homogenous solution (Houbenweyl, 1987, Methods of Organic Chemistry, ed. E. Wansch, Vol. 15 I and II, Thieme, Stuttgart).

III. Uses

The present invention includes all uses of the nucleic acid molecules, TCAP peptides and preTCAP peptides of the invention including, but not limited to, the preparation of antibodies and antisense oligonucleotides, the preparation of experimental systems to study TCAP, the isolation of substances that can bind or modulate TCAP expression and/or activity as well as the use of the TCAP nucleic acid sequences and peptides and modulators thereof in diagnostic and therapeutic applications. Some of the uses are further described below.

(a) Antibodies

The isolation of the TCAP peptide enables the preparation of antibodies specific for TCAP. Accordingly, the present invention provides an antibody that binds to a TCAP peptide and/or a protein containing a TCAP peptide, such as preTCAP.

Conventional methods can be used to prepare the antibodies. For example, by using a TCAP, polyclonal antisera or monoclonal antibodies can be made using standard methods. A mammal, (e.g., a mouse, hamster, or rabbit) can be immunized with an immunogenic form of the peptide which elicits an antibody response in the mammal. Techniques for conferring immunogenicity on a peptide include conjugation to carriers or other techniques well known in the art. For example, the protein or peptide can be administered in the presence of adjuvant. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other immunoassay procedures can be used with the immunogen as antigen to assess the levels of antibodies. Following immunization, antisera can be obtained and, if desired, polyclonal antibodies isolated from the sera.

To produce monoclonal antibodies, antibody-producing cells (lymphocytes) can be harvested from an immunized animal and fused with myeloma cells by standard somatic cell fusion procedures thus immortalizing these cells and yielding hybridoma cells. Such techniques are well known in the art, (e.g., the hybridoma technique originally developed by Kohler and Milstein (Nature 256, 495-497 (1975)) as well as other techniques such as the human B-cell hybridoma technique (Kozbor et al., Immunol. Today 4, 72 (1983)), the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al. Monoclonal Antibodies in Cancer Therapy (1985) Allen R. Bliss, Inc., pages 77-96), and screening of combinatorial antibody libraries (Huse et al., Science 246, 1275 (1989)). Hybridoma cells can be screened immunochemically for production of antibodies specifically reactive with the peptide and the monoclonal antibodies can be isolated. Therefore, the invention also contemplates hybridoma cells secreting monoclonal antibodies with specificity for TCAP.

The term "antibody" as used herein is intended to include fragments thereof which also specifically react with TCAP. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner as described above. For example, F(ab')2 fragments can be generated by treating antibody with pepsin. The resulting F(ab')2 fragment can be further treated to produce Fab' fragments.

Chimeric antibody derivatives, i.e., antibody molecules that combine a non-human animal variable region and a human constant region are also contemplated within the scope of the invention. Chimeric antibody molecules can include, for example, the antigen binding domain from an antibody of a mouse, rat, or other species, with human constant regions. Conventional methods may be used to make chimeric antibodies containing the immunoglobulin variable region which recognizes the gene product of TCAP antigen of the invention (See, for example, Morrison et al., Proc. Natl Acad. Sci. U.S.A. 81,6851 (1985); Takeda et al., Nature 314, 452 (1985), Cabilly et al., U.S. Patent No. 4,816,567; Boss et al., U.S. Patent No. 4,816,397; Tanaguchi et al., European Patent Publication EP171496; European Patent

Publication 0173494, United Kingdom patent GB 2177096B). It is expected that chimeric antibodies would be less immunogenic in a human subject than the corresponding non-chimeric antibody.

Monoclonal or chimeric antibodies specifically reactive with a peptide of the invention as described herein can be further humanized by producing human constant region chimeras, in which parts of the variable regions, particularly the conserved framework regions of the antigen-binding domain, are of human origin and only the hypervariable regions are of non-human origin. Such immunoglobulin molecules may be made by techniques known in the art, (e.g., Teng et al., Proc. Natl. Acad. Sci. U.S.A., 80, 7308-7312 (1983); Kozbor et al., Immunology Today, 4, 7279 (1983); Olsson et al., Meth. Enzymol., 92, 3-16 (1982)), and PCT Publication WO92/06193 or EP 0239400). Humanized antibodies can also be commercially produced (Scotgen Limited, 2 Holly Road, Twickenham, Middlesex, Great Britain.)

Specific antibodies, or antibody fragments, reactive against TCAP peptide may also be generated by screening expression libraries encoding immunoglobulin genes, or portions thereof, expressed in bacteria with peptides produced from the nucleic acid molecules encoding TCAP. For example, complete Fab fragments, VH regions and FV regions can be expressed in bacteria using phage expression libraries (See for example Ward et al., Nature 341, 544-546: (1989); Huse et al., Science 246, 1275-1281 (1989); and McCafferty et al. Nature 348, 552-554 (1990)). Alternatively, a SCID-hu mouse, for example the model developed by Genpharm, can be used to produce antibodies or fragments thereof.

(b) Antisense Oligonucleotides

Isolation of a nucleic acid molecule encoding TCAP enables the production of antisense oligonucleotides that can modulate the expression and/or activity of TCAP. Accordingly, the present invention provides an antisense oligonucleotide that is complimentary to a nucleic acid sequence encoding TCAP.

The term "antisense oligonucleotide" as used herein means a nucleotide sequence that is complimentary to its target.

The term "oligonucleotide" refers to an oligomer or polymer of nucleotide or nucleoside monomers consisting of naturally occurring bases, sugars, and intersugar (backbone) linkages. The term also includes modified or substituted oligomers comprising non-naturally occurring monomers or portions thereof, which function similarly. Such modified or substituted oligonucleotides may be preferred over naturally occurring forms because of properties such as enhanced cellular uptake, or increased stability in the presence of nucleases. The term also includes chimeric oligonucleotides which contain two or more chemically distinct regions. For example, chimeric oligonucleotides may contain at least one region of modified nucleotides that confer beneficial properties (e.g. increased nuclease resistance, increased uptake into cells), or two or more oligonucleotides of the invention may be joined to form a chimeric oligonucleotide.

The antisense oligonucleotides of the present invention may be ribonucleic or deoxyribonucleic acids and may contain naturally occurring bases including adenine, guanine, cytosine, thymidine and uracil. The oligonucleotides may also contain modified bases such as xanthine, hypoxanthine, 2-aminoadenine, 6-methyl, 2-propyl and other alkyl adenines, 5-halo uracil, 5-halo cytosine, 6-aza uracil, 6-aza cytosine and 6-aza thymine, pseudo uracil, 4-thiouracil, 8-halo adenine, 8-aminoadenine, 8-thiol adenine, 8-thiolalkyl adenines, 8-hydroxyl adenine and other 8-substituted adenines, 8-halo guanines, 8-amino guanine, 8-thiol guanine, 8-thiolalkyl guanines, 8-hydroxyl guanine and other 8-substituted guanines, other aza and deaza uracils, thymidines, cytosines, adenines, or guanines, 5-trifluoromethyl uracil and 5-trifluoro cytosine.

Other antisense oligonucleotides of the invention may contain modified phosphorous, oxygen heteroatoms in the phosphate backbone, short chain alkyl or cycloalkyl intersugar linkages or short chain heteroatomic or heterocyclic intersugar linkages. For example, the antisense oligonucleotides may contain phosphorothioates, phosphotriesters, methyl phosphonates, and phosphorodithioates. In an embodiment of the invention there are

phosphorothioate bonds links between the four to six 3'-terminus bases. In another embodiment phosphorothioate bonds link all the nucleotides.

The antisense oligonucleotides of the invention may also comprise nucleotide analogs that may be better suited as therapeutic or experimental reagents. An example of an oligonucleotide analogue is a peptide nucleic acid (PNA) wherein the deoxyribose (or ribose) phosphate backbone in the DNA (or RNA), is replaced with a polyamide backbone which is similar to that found in peptides (P.E. Nielsen, et al Science 1991, 254, 1497). PNA analogues have been shown to be resistant to degradation by enzymes and to have extended lives *in vivo* and *in vitro*. PNAs also bind stronger to a complimentary DNA sequence due to the lack of charge repulsion between the PNA strand and the DNA strand. Other oligonucleotides may contain nucleotides containing polymer backbones, cyclic backbones, or acyclic backbones. For example, the nucleotides may have morpholino backbone structures (U.S. Pat. No. 5,034,506). Oligonucleotides may also contain groups such as reporter groups, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an antisense oligonucleotide. Antisense oligonucleotides may also have sugar mimetics.

The antisense nucleic acid molecules may be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. The antisense nucleic acid molecules of the invention or a fragment thereof, may be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed with mRNA or the native gene e.g. phosphorothioate derivatives and acridine substituted nucleotides. The antisense sequences may be produced biologically using an expression vector introduced into cells in the form of a recombinant plasmid, phagemid or attenuated virus in which antisense sequences are produced under the control of a high efficiency regulatory region, the activity of which may be determined by the cell type into which the vector is introduced.

The antisense oligonucleotides may be introduced into tissues or cells using techniques in the art including vectors (retroviral vectors, adenoviral vectors and DNA virus vectors) or physical techniques such as microinjection. The antisense oligonucleotides may be directly administered *in vivo* or may be used to transfect cells *in vitro* which are then administered *in vivo*. In one embodiment, the antisense oligonucleotide may be delivered to macrophages and/or endothelial cells in a liposome formulation.

(c) Diagnostic Assays

The findings by the present inventors that TCAP is involved in inhibiting neuronal cell proliferation, in inducing an anxiogenic response and in inhibiting cell death in cells subject to stress allows development of diagnostic assays, particularly for conditions associated with the aberrant regulation of neuronal growth.

Accordingly, the present invention provides a method of detecting a condition associated with TCAP or preTCAP expression comprising assaying a sample for (a) a nucleic acid molecule encoding a TCAP peptide or a fragment thereof or (b) a TCAP protein or a fragment thereof. The TCAP peptide preferably has a sequence as shown in SEQ.ID.NOS.: 13, 14, 21, 22, 29, 30, 37, 38, 45, 46, 53, 54, 61, 62, 69, 70, 77, 78, 85, 86, 93, 94, 101, 103. In one particular embodiment of the invention the condition is associated with the aberrant regulation of neuronal growth. Neuronal growth may include somatic and process development, mitogenesis or migration. Aberrant regulation of neuronal growth may occur via a disturbance in interneuronal connections and the associated signal molecules. Examples of such conditions include learning deficits, mental retardation, autism, schizophrenia, Alzheimer's Disease, Parkinson's Disease as well as affective disorders such as panic disorder, depression, anorexia nervosa and obsessive-compulsive disorder.

(i) Nucleic acid molecules

The nucleic acid molecules encoding TCAP as described herein or fragments thereof, allow those skilled in the art to construct nucleotide probes for use in the detection of nucleotide sequences encoding TCAP or fragments

thereof in samples, preferably biological samples such as cells, tissues and bodily fluids. The probes can be useful in detecting the presence of a condition associated with TCAP expression or monitoring the progress of such a condition. Accordingly, the present invention provides a method for detecting a nucleic acid molecule encoding a TCAP comprising contacting the sample with a nucleotide probe capable of hybridizing with the nucleic acid molecule to form a hybridization product, under conditions which permit the formation of the hybridization product, preferably under stringent conditions, and assaying for the hybridization product.

Example of probes that may be used in the above method include fragments of the nucleic acid sequences shown in SEQ.ID.NOS.:-18-20, 25-28, 33-36, 41-44, 49-52, 57-60, 65-68, 73-76, 81-84, 89-92, 97-100 or that wherein T can also be U or that encodes a peptide having an amino acid sequence selected from the group consisting of : SEQ. ID. NOS: 13, 14, 21, 22, 29, 30, 37, 38, 45, 46, 53, 54, 61, 62, 69, 70, 77, 78, 85, 86, 93, 94, 101, 103 or that further has an amidation signal sequence (preferably GKR or GRR), at the carboxy terminus of said peptides, such as 15, 16, 23, 24, 31, 32, 39, 40, 47, 48, 55, 56, 63, 64, 71, 72, 79, 80, 97, 88, 95, 96. A nucleotide probe may be labelled with a detectable substance such as a radioactive label which provides for an adequate signal and has sufficient half-life such as 32P, 3H, 14C or the like. Other detectable substances which may be used include antigens that are recognized by a specific labelled antibody, fluorescent compounds, enzymes, antibodies specific for a labelled antigen, and chemiluminescence. An appropriate label may be selected having regard to the rate of hybridization and binding of the probe to the nucleic acid to be detected and the amount of nucleic acid available for hybridization. Labelled probes may be hybridized to nucleic acids on solid supports such as nitrocellulose filters or nylon membranes as generally described in Sambrook et al, 1989, Molecular Cloning, A Laboratory Manual (2nd ed.). The nucleotide probes may be used to detect genes, preferably in human cells, that hybridize to the nucleic acid molecule of the present invention preferably,

nucleic acid molecules which hybridize to the nucleic acid molecule of the invention under stringent hybridization conditions as described herein.

Nucleic acid molecules encoding a TCAP peptide can be selectively amplified in a sample using the polymerase chain reaction (PCR) methods and cDNA or genomic DNA. It is possible to design synthetic oligonucleotide primers from the nucleotide sequence shown in Figures 1 – 5 .for use in PCR. A nucleic acid can be amplified from cDNA or genomic DNA using oligonucleotide primers and standard PCR amplification techniques. The amplified nucleic acid can be cloned into an appropriate vector and characterized by DNA sequence analysis. cDNA may be prepared from mRNA, by isolating total cellular mRNA by a variety of techniques, for example, by using the guanidinium-thiocyanate extraction procedure of Chirgwin et al., Biochemistry, 18, 5294-5299 (1979). cDNA is then synthesized from the mRNA using reverse transcriptase (for example, Moloney MLV reverse transcriptase available from Gibco/BRL, Bethesda, MD, or AMV reverse transcriptase available from Seikagaku America, Inc., St. Petersburg, FL).

Patients may be screened routinely using probes to detect the presence of a TCAP gene by a variety of techniques. Genomic DNA used for the diagnosis may be obtained from body cells, such as those present in the blood, tissue biopsy, surgical specimen, or autopsy material. The DNA may be isolated and used directly for detection of a specific sequence or may be PCR amplified prior to analysis. RNA or cDNA may also be used. To detect a specific DNA sequence hybridization using specific oligonucleotides, direct DNA sequencing, restriction enzyme digest, RNase protection, chemical cleavage, and ligase-mediated detection are all methods which can be utilized. Oligonucleotides specific to mutant sequences can be chemically synthesized and labelled radioactively with isotopes, or non-radioactively using biotin tags, and hybridized to individual DNA samples immobilized on membranes or other solid-supports by dot-blot or transfer from gels after electrophoresis. The presence or absence of these mutant sequences is then visualized using methods such as autoradiography, fluorometry, or colorimetric reaction.

Suitable PCR primers can be generated which are useful for example in amplifying portions of the subject sequence containing identified mutations. Other nucleotide sequence amplification techniques may be used, such as ligation-mediated PCR, anchored PCR and enzymatic amplification as would be understood by those skilled in the art.

Sequence alterations may also generate fortuitous restriction enzyme recognition sites that are revealed by the use of appropriate enzyme digestion followed by gel-blot hybridization. DNA fragments carrying the site (normal or mutant) are detected by their increase or reduction in size, or by the increase or decrease of corresponding restriction fragment numbers. Genomic DNA samples may also be amplified by PCR prior to treatment with the appropriate restriction enzyme and the fragments of different sizes are visualized under UV light in the presence of ethidium bromide after gel electrophoresis.

Genetic testing based on DNA sequence differences may be achieved by detection of alteration in electrophoretic mobility of DNA fragments in gels. Small sequence deletions and insertions can be visualized by high-resolution gel electrophoresis. Small deletions may also be detected as changes in the migration pattern of DNA heteroduplexes in non-denaturing gel electrophoresis. Alternatively, a single base substitution mutation may be detected based on differential primer length in PCR. The PCR products of the normal and mutant gene could be differentially detected in acrylamide gels.

Nuclease protection assays (S1 or ligase-mediated) also reveal sequence changes at specific locations. Alternatively, to confirm or detect a polymorphism restriction mapping changes ligated PCR, ASO, REF-SSCP and SSCP may be used. Both REF-SSCP and SSCP are mobility shift assays that are based upon the change in conformation due to mutations.

DNA fragments may also be visualized by methods in which the individual DNA samples are not immobilized on membranes. The probe and target sequences may be in solution or the probe sequence may be immobilized. Autoradiography, radioactive decay, spectrophotometry, and fluorometry may also be used to identify specific individual genotypes.

(ii) Proteins

The TCAP protein may be detected in a sample using antibodies that bind to the protein as described in detail above. Accordingly, the present invention provides a method for detecting a TCAP protein comprising contacting the sample with an antibody that binds to TCAP and which is capable of being detected after it becomes bound to the TCAP in the sample.

Antibodies specifically reactive with TCAP, or derivatives thereof, such as enzyme conjugates or labeled derivatives, may be used to detect TCAP in various biological materials, for example they may be used in any known immunoassays which rely on the binding interaction between an antigenic determinant of TCAP, and the antibodies. Examples of such assays are radioimmunoassays, enzyme immunoassays (e.g. ELISA), immunofluorescence, immunoprecipitation, latex agglutination, hemagglutination and histochemical tests. Thus, the antibodies may be used to detect and quantify mutated TCAP in a sample in order to determine its role in particular cellular events or pathological states, and to diagnose and treat such pathological states.

In particular, the antibodies of the invention may be used in immuno-histochemical analyses, for example, at the cellular and sub-subcellular level, to detect TCAP, to localize it to particular cells and tissues and to specific subcellular locations, and to quantitate the level of expression.

Cytochemical techniques known in the art for localizing antigens using light and electron microscopy may be used to detect TCAP. Generally, an antibody of the invention may be labelled with a detectable substance and TCAP may be localised in tissue based upon the presence of the detectable substance. Examples of detectable substances include various enzymes, fluorescent materials, luminescent materials and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, biotin, alkaline phosphatase, β-galactosidase, or acetylcholinesterase; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; and examples of suitable radioactive material include radioactive iodine I-125, I-

131 or 3-H. Antibodies may also be coupled to electron dense substances, such as ferritin or colloidal gold, which are readily visualized by electron microscopy.

Indirect methods may also be employed in which the primary antigen-antibody reaction is amplified by the introduction of a second antibody, having specificity for the antibody reactive against TCAP. By way of example, if the antibody having specificity against TCAP is a rabbit IgG antibody, the second antibody may be goat anti-rabbit gamma-globulin labelled with a detectable substance as described herein.

Where a radioactive label is used as a detectable substance, TCAP may be localized by autoradiography. The results of autoradiography may be quantitated by determining the density of particles in the autoradiographs by various optical methods, or by counting the grains.

(d) Experimental Systems

Eukaryotic expression systems are preferred and can be used for many studies of TCAP encoding genes and gene product(s) including the production of large amounts of the peptide for isolation and purification, to use cells expressing the TCAP peptide as a functional assay system for antibodies generated against the peptide or to test effectiveness of pharmacological agents, to study the function of the normal complete peptide, specific portions of the peptide, or of naturally occurring and artificially produced mutant peptides.

Using the techniques mentioned, the expression vectors containing the TCAP peptide cDNA sequence or portions thereof can be introduced into a variety of mammalian cells from other species or into non-mammalian cells.

The recombinant cloning vector, according to this invention, comprises the selected DNA of the DNA sequences of this invention for expression in a suitable host. The DNA is operatively linked in the vector to an expression control sequence in the recombinant DNA molecule so that TCAP peptide protein can be expressed. The expression control sequence may be selected from the group consisting of sequences that control the expression of genes of eukaryotic cells and their viruses and combinations thereof. The expression

control sequence may be selected from the group consisting of the lac system, the trp system, the tac system, the trc system, major operator and promoter regions of phage lambda, the control region of the fd coat protein, early and late promoters of TCAP, promoters derived from polyoma, adenovirus, retrovirus, baculovirus, simian virus, 3-phosphoglycerate kinase promoter, yeast acid phosphatase promoters, yeast alpha-mating factors and combinations thereof.

Expression of the TCAP peptide in heterologous cell systems may also be used to demonstrate structure-function relationships as well as to provide cell lines for the purposes of drug screening. Inserting a TCAP DNA sequence into a plasmid expression vector to transfect cells is a useful method to test the influence of the peptide on various cellular biochemical parameters including the identification of substrates as well as activators and inhibitors of the gene. Plasmid expression vectors containing either the entire coding sequence for TCAP, or for portions thereof, can be used in *in vitro* mutagenesis experiments that will identify portions of the protein crucial for function. The DNA sequence can be manipulated in studies to understand the expression of the gene and its product. The changes in the sequence may or may not alter the expression pattern in terms of relative quantities, tissue-specificity and functional properties.

The invention also provides methods for examining the function of the TCAP peptide encoded by the nucleic acid molecules of the invention. Cells, tissues, and non-human animals lacking in expression or partially lacking in expression of the peptide may be developed using recombinant molecules of the invention having specific deletion or insertion mutations in the nucleic acid molecule of the invention. A recombinant molecule may be used to inactivate or alter the endogenous gene by homologous recombination, and thereby create a deficient cell, tissue or animal. Such a mutant cell, tissue or animal may be used to define specific cell populations, developmental patterns and *in vivo* processes, normally dependent on the protein encoded by the nucleic acid molecule of the invention.

Immortalized TCAP responsive cell lines can also be used to identify modulators of TCAP such as noted in Example 13. It can also be used to identify effect of TCAP and TCAP modulators on particular markers. In so far as these markers are associated with the regulation of a medical condition, TCAP and/or the TCAPmodulators may be used in the diagnosis, regulation, and/or treatment of said medical condition.

(e) TCAP Modulators

In addition to antibodies and antisense oligonucleotides described above, other substances that modulate TCAP expression or activity may also be identified.

(i) Substances that Bind/ModulateTCAP

Substances that affect TCAP activity can be identified based on their ability to bind to TCAP.

Substances which can bind with the TCAP of the invention may be identified by reacting the TCAP with a substance which potentially binds to TCAP, and assaying for complexes, for free substance, or for non-complexed TCAP, or for activation of TCAP. In particular, a yeast two hybrid assay system may be used to identify proteins which interact with TCAP (Fields, S. and Song, O., 1989, Nature, 340:245-247). Systems of analysis which also may be used include ELISA.

Accordingly, the invention provides a method of identifying substances which can bind with TCAP, comprising the steps of:

1. reacting TCAP and a test substance, under conditions which allow for formation of a complex between the TCAP and the test substance, and
2. assaying for complexes of TCAP and the test substance, for free substance or for non complexed TCAP, wherein the presence of complexes indicates that the test substance is capable of binding TCAP.

In another embodiment the invention provides a method of identifying substances that can modulate TCAP activity, such as by binding to TCAP or a TCAP substrate and thus potentially compete (i.e. inhibit TCAP activity), or enhance TCAP/substrate interaction (i.e enhancing TCAP activity), the method comprising:

1. reacting TCAP and a TCAP substate and a test substance, under conditions which allow for formation of a complex between the TCAP and the TCAP substrate, and
2. assaying for complexes of TCAP and the test substance, TCAP and TCAP substate, TCAP substrate and test substance, for free substance or for non complexed TCAP or TCAP substrate, wherein the presence of complexes with the test substance indicates that the test substance is capable of binding TCAP or TCAP substrate, as the case may be.

In another embodiment, a method of identifying modulators of TCAP comprises the use of a cell line that has known reaction to TCAP that can be monitored and monitoring said reaction in the presence of TCAP and a potential modulator.

The TCAP peptide used in the assay may have the amino acid sequence shown in SEQ.ID.NOS:, 14, 21, 22, 29, 30, 37, 38, 45, 46, 53, 54, 61, 62, 69, 70, 77, 78, 85, 86, 93, 94, 101, 103 or may be a fragment, analog, derivative, homolog or mimetic thereof as described herein.

Conditions which permit the formation of substance and TCAP complexes may be selected having regard to factors such as the nature and amounts of the substance and the peptide.

The substance-peptide complex, free substance or non-complexed peptides may be isolated by conventional isolation techniques, for example, salting out, chromatography, electrophoresis, gel filtration, fractionation, absorption, polyacrylamide gel electrophoresis, agglutination, or combinations thereof. To facilitate the assay of the components, antibody against TCAP or the substance, or labelled TCAP, or a labelled substance may be utilized. The antibodies, proteins, or substances may be labelled with a detectable substance as described above.

TCAP, or the substance used in the method of the invention may be insolubilized. For example, TCAP or substance may be bound to a suitable carrier. Examples of suitable carriers are agarose, cellulose, dextran, Sephadex, Sepharose, carboxymethyl cellulose polystyrene, filter paper, ion-

exchange resin, plastic film, plastic tube, glass beads, polyamine-methyl vinyl-ether-maleic acid copolymer, amino acid copolymer, ethylene-maleic acid copolymer, nylon, silk, etc. The carrier may be in the shape of, for example, a tube, test plate, beads, disc, sphere etc.

The insolubilized peptide or substance may be prepared by reacting the material with a suitable insoluble carrier using known chemical or physical methods, for example, cyanogen bromide coupling.

The peptide or substance may also be expressed on the surface of a cell using the methods described herein.

The invention also contemplates assaying for an antagonist or agonist of the action of TCAP.

It will be understood that the agonists and antagonists that can be assayed using the methods of the invention may act on one or more of the binding sites on the protein or substance including agonist binding sites, competitive antagonist binding sites, non-competitive antagonist binding sites or allosteric sites.

The invention also makes it possible to screen for antagonists that inhibit the effects of an agonist of TCAP. Thus, the invention may be used to assay for a substance that competes for the same binding site of TCAP.

(ii) Peptide Mimetics

The present invention also includes peptide mimetics of TCAP. "Peptide mimetics" are structures which serve as substitutes for peptides in interactions between molecules (See Morgan et al (1989), Ann. Reports Med. Chem. 24:243-252 for a review). Peptide mimetics include synthetic structures which may or may not contain amino acids and/or peptide bonds but retain the structural and functional features of a peptide, or enhancer or inhibitor of the invention. Peptide mimetics also include peptoids, oligopeptoids (Simon et al (1972) Proc. Natl. Acad, Sci USA 89:9367); and peptide libraries containing peptides of a designed length representing all possible sequences of amino acids corresponding to a peptide of the invention.

Peptide mimetics may be designed based on information obtained by systematic replacement of L-amino acids by D-amino acids, replacement of

side chains with groups having different electronic properties, and by systematic replacement of peptide bonds with amide bond replacements. Local conformational constraints can also be introduced to determine conformational requirements for activity of a candidate peptide mimetic. The mimetics may include isosteric amide bonds, or D-amino acids to stabilize or promote reverse turn conformations and to help stabilize the molecule. Cyclic amino acid analogues may be used to constrain amino acid residues to particular conformational states. The mimetics can also include mimics of inhibitor peptide secondary structures. These structures can model the 3-dimensional orientation of amino acid residues into the known secondary conformations of proteins. Peptoids may also be used which are oligomers of N-substituted amino acids and can be used as motifs for the generation of chemically diverse libraries of novel molecules.

Peptides of the invention may also be used to identify lead compounds for drug development. The structure of the peptides described herein can be readily determined by a number of methods such as NMR and X-ray crystallography. A comparison of the structures of peptides similar in sequence, but differing in the biological activities they elicit in target molecules can provide information about the structure-activity relationship of the target. Information obtained from the examination of structure-activity relationships can be used to design either modified peptides, or other small molecules or lead compounds that can be tested for predicted properties as related to the target molecule. The activity of the lead compounds can be evaluated using assays similar to those described herein.

Information about structure-activity relationships may also be obtained from co-crystallization studies. In these studies, a peptide with a desired activity is crystallized in association with a target molecule, and the X-ray structure of the complex is determined. The structure can then be compared to the structure of the target molecule in its native state, and information from such a comparison may be used to design compounds expected to possess.

(iii) Drug Screening Methods

In accordance with one embodiment, the invention enables a method for screening candidate compounds for their ability to increase or decrease the activity and/or expression of TCAP. The method comprises providing an assay system for assaying TCAP activity, assaying the activity in the presence or absence of the candidate or test compound and determining whether the compound has increased or decreased TCAP activity. Such compounds may be useful in treating conditions associated with aberrant regulation of neuronal growth.

Accordingly, the present invention provides a method for identifying a compound that affects TCAP activity or expression comprising:

(a) incubating a test compound with a TCAP peptide or a nucleic acid encoding a TCAP peptide; and

(b) determining an amount of TCAP peptide activity or expression and comparing with a control (i.e. in the absence of the test substance), wherein a change in the TCAP activity or expression as compared to the control indicates that the test compound has an effect on TCAP activity or expression.

In accordance with a further embodiment, the invention enables a method for screening candidate compounds for their ability to increase or decrease expression of a TCAP peptide. The method comprises putting a cell with a candidate compound, wherein the cell includes a regulatory region of a gene encoding TCAP operably joined to a reporter gene coding region, and detecting a change in expression of the reporter gene.

Such compounds can be selected from protein compounds, chemicals and various drugs that are added to the culture medium. After a period of incubation in the presence of a selected test compound(s), the expression of mutated TCAP can be examined by quantifying the levels of TCAP mRNA using standard Northern blotting procedure, as described in the examples included herein, to determine any changes in expression as a result of the test compound. Cell lines transfected with constructs expressing TCAP can also be used to test the function of compounds developed to modify the protein expression.

(f) Therapeutic Uses

As previously discussed, TCAP of the invention is involved in cAMP, cGMP activity, neuronal growth and neurological development. Accordingly, the present invention provides a method of treating a condition associated with aberrant regulation of cAMP, cGMP, neuronal growth, neuronal communciation, or neuronal cell proliferation comprising the administering to a cell or animal in need thereof, an effective amount of agent that modulates TCAP expression and/or activity.

The term "agent that modulates TCAP expression and/or activity" means any substance that can alter the expression and/or activity of TCAP. Examples of agents which may be used to in administration include: a nucleic acid molecule encoding TCAP; the TCAP peptide as well as fragments, analogs, derivatives or homologs thereof; antibodies; antisense nucleic acids; peptide mimetics; and substances isolated using the screening methods described herein that can result in TCAP levels and/or function consistent with a person without the condition.

The term "effective amount" as used herein means an amount effective, at dosages and for periods of time necessary to achieve the desired results.

The term "animal" as used herein includes all members of the animal kingdom that respond to TCAP, preferably mammals, including both human and non-human animals, more preferably humans. In another embodiment, animals include domesticated animals, such as cows, horses, pigs, and sheep, In another embodiment, the animals are from the avian family and include chickens.

In accordance with another embodiment, the present invention enables gene therapy as a potential therapeutic approach to a condition, in which normal copies of the TCAP gene are introduced into patients to successfully code for normal TCAP peptide in several different affected cell types.

Retroviral vectors can be used for somatic cell gene therapy especially because of their high efficiency of infection and stable integration and expression. The targeted cells however must be able to divide and the expression of the levels of normal protein or peptide should be high. A TCAP

encoding gene can be cloned into a retroviral vector and driven from its endogenous promoter or from the retroviral long terminal repeat or from a promoter specific for the target cell type of interest (such as lymphoid cells). Other viral vectors that can be used include adeno-associated virus, vaccinia virus, bovine papilloma virus, or a herpesvirus such as Epstein-Barr virus. Gene transfer could also be achieved using non-viral means requiring infection *in vitro*. This would include calcium phosphate, DEAE dextran, electroporation, cationic or anionic lipid formulations (liposomes) and protoplast fusion. Although these methods are available, many of these are lower efficiency.

Anti-sense based strategies can be employed to inhibit TCAP gene function and as a basis for therapeutic drug design. The principle is based on the hypothesis that sequence specific suppression of gene expression can be achieved by intracellular hybridization between mRNA and a complementary anti-sense species. It is possible to synthesize anti-sense strand nucleotides that bind the sense strand of RNA or DNA with a high degree of specificity. The formation of a hybrid RNA duplex may interfere with the processing/transport/translation and/or stability of a target mRNA.

Hybridization is required for an antisense effect to occur. Antisense effects have been described using a variety of approaches including the use of antisense oligonucleotides, injection of antisense RNA, DNA and transfection of antisense RNA expression vectors.

Therapeutic antisense nucleotides can be made as oligonucleotides or expressed nucleotides. Oligonucleotides are short single strands of DNA which are usually 15 to 20 nucleic acid bases long. Expressed nucleotides are made using expression vectors such as an adenoviral, retroviral or plasmid vector. The vector is administered to the cells in culture, or to a patient, whose cells then make the antisense nucleotide. Expression vectors can be designed to produce antisense RNA, which can vary in length from a few dozen bases to several thousand.

Antisense effects can be induced by control (sense) sequences. The extent of phenotypic changes is highly variable. Phenotypic effects induced by

antisense are based on changes in criteria such as biological endpoints, protein levels, protein activation measurement and target mRNA levels.

(g) Methods And Uses Of TCAP For Modulation Of Stress Responses, Related Conditions And Anxiety

The invention also provides a method of detecting an anxiety disorder in an animal by monitoring the effect of TCAP on said animal. If the anxiety response decreases (anxiolytic) as compared to baseline level, than the animal may have a high anxiety related disorder. If the anxiety response of an animal increases in response to administration of TCAP, then the animal may have a low anxiety disorder.

The invention provides a method for normalizing the anxiety state of an animal by administering TCAP to said animal or up-regulating TCAP expression in said animal.

The invention also provides a method of inducing a desired anxiety state in an animal by:

(a) determining whether the animal is a low or high anxiety animal; and

(b) (i) administering an effective amount of TCAP or TCAP agonist (including a substance or nucleic acid molecule that up regulates TCAP expression) to increase anxiety in a low anxiety animal and decrease anxiety in a high anxiety animal; or

(ii) administering an inhibitor of TCAP or TCAP antagonist (including a substance or nucleic acid molecule, such as a TCAP antisense nucleic acid molecule, that down regulates TCAP expression) to increase anxiety in a high anxiety animal and decrease anxiety in a low anxiety animal.

The invention also provides a method of detecting a modulator of TCAP activity comprising, administering TCAP to an animal with a known anxiety state (high or low anxiety), administering the potential modulator to said animal and comparing the response to TCAP in the presence and absence of said substance. If the animal's response to TCAP is different than that of baseline (Animal with TCAP alone, and no substance), then said substance is a

modulator of TCAP activity. Such compounds may be used to treat animals with undesired stress or anxiety levels.

In one embodiment, TCAP is TCAP-1, or analog, derivative or fragment thereof with similar biological activity.

In another embodiment a modulator of TCAP is administered to modulate or regulate the stress response in an animal.

Stress as used herein is any state that is not homeostasis or metabolic balance. Stress is also used to refer to the general state of stressors provoking stress responses (Sapolsky, 1992). Hoemeostasis refers to the normal stability of the internal environment (Sapolsky, 1992). A Stressor is defined as anything that disrupts physiological balance, be it physical or psychological (Sapolsky, 1992). For example, a stressor in the behavioural experimentals herein (Examples 10 and 11)is defined as a 120 dB tone using the acoustic startle test.

Stress Response as used herein is a physiological or behavioural response to stressor(s). For example, in the behavioural experiments (Examples 10 and 11), stress response is the startle response as measured by the acoustic startle testing apparatus (Med Associates, St. Albans, VT) following presentation of a 120 dB tone.

Anxiogenic as used herein means a stimulus, internal or external, that increases behavioural measures of anxiety in generally accepted tests. In Examples 10 and 11 herein, the behavioural measure of anxiety is the startle response as measured by the acoustic startle testing apparatus (Med Associates, St. Albans, VT) following the presentation of a 120 dB tone. An anxiogenic response is an increase in the startle response.

Anxiolytic as used herein means a stimulus, internal or external, that decreases behavioural measures of anxiety in generally accepted tests. . In Examples 10 and 11 herein, the behavioural measure of anxiety is the startle response as measured by the acoustic startle testing apparatus (Med Associates, St. Albans, VT) following the presentation of a 120 dB tone. An anxiolytic response is a decrease in the startle response.

Anxiety refers to a generalized state of distress that may be prompted by generalized, non-specific cues, and involves physiological arousal, but often without organized functional behaviour (Lang et al., 2000). Animal models of anxiety attempt to represent some aspect of the etiology, symptomatology, or treatment of these disorders (Menard and Treit, 1999). In the present studies, the acoustic startle response was used as a measure of anxiety (Frankland et al., 1996, 1997). This test measures a simple reflex induced by a loud and unexpected auditory stimulus, and can be measured using standardized equipment (Med Associates, St. Albans, Vermont).

High Anxiety as used herein means an animal,e.g., rat, that has a post-vehicle injection startle response that is greater than the baseline response. An average startle response is calculated for the baseline trials and the post-injection (treatment) test periods. The treatment/baseline ratio is then calculated for each animal, e.g., rat. If this ratio is greater than 1, then the animal is classified as high anxiety.

Low Anxiety as used herein means an animal, e.g rat, that has a post-vehicle injection startle response that is less than the baseline response. The treatment/baseline ratio is calculated for each animal, e.g. rat ,as above. If this ratio is less than 1, then the animal, e.g. rat, is classified as low anxiety.

Normal Anxiety as used herein means an animal, such as a rat that has a post-vehicle injection startle response that is the same as the baseline response. The treatment/baseline ratio is calculated for each rat as above. If this ratio is equal to 1, then the animal, e.g.rat, is classified as normal anxiety.

(h) The Role Of TCAP In The Regulation of Cell Proliferation and in the Treatment of Cancer

In one embodiment, the invention provides a method of regulating cell proliferation by administering an effective amount of TCAP to an animal in need thereof. In another embodiment, the TCAP is administered *in vivo* or *in vitro* to decreasing and/or inhibiting cell proliferation. In one embodiment the cell is cancerous. In another embodiment the cell is a neuronal tumour cell.

In one embodiment, TCAP or modulators thereof can be used in the treatment of cancer, such as neuroblastomas or other neuronal tumours.

(i) **Pharmaceutical Compositions**

The above described substances including nucleic acids encoding TCAP, TCAP peptides, antibodies, and antisense oligonucleotides as well as other agents that modulate TCAP activity or expression may be formulated into pharmaceutical compositions for administration to subjects in a biologically compatible form suitable for administration *in vivo*. By "biologically compatible form suitable for administration *in vivo*" is meant a form of the substance to be administered in which any toxic effects are outweighed by the therapeutic effects. The substances may be administered to living organisms including humans, and animals.

Thus in one embodment, the invention provides the use of TCAp or modulator there in the preparation of a medicament for the treatment of TCAP –related or TCAP regulated medical conditions. For instance, in the regulation of cell proliferation (e.g. cancer), stress, anxiety or neuronal communicative disorders.

Administration of a therapeutically effective amount of pharmaceutical compositions of the present invention is defined as an amount effective, at dosages and for periods of time necessary to achieve the desired therapeutic result. For example, a therapeutically effective amount of a substance may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the substance to elicit a desired response in the individual. Dosage regimes may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

An active substance may be administered in a convenient manner such as by injection (subcutaneous, intravenous, etc.), oral administration, inhalation, transdermal application, or rectal administration. Depending on the route of administration, the active substance may be coated in a material to protect the compound from the action of enzymes, acids and other natural

conditions that may inactivate the compound. If the active substance is a nucleic acid encoding, for example, a TCAP peptide it may be delivered using techniques known in the art.

The compositions described herein can be prepared by *per se* known methods for the preparation of pharmaceutically acceptable compositions which can be administered to subjects, such that an effective quantity of the active substance is combined in a mixture with a pharmaceutically acceptable vehicle. Suitable vehicles are described, for example, in Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., USA 1985) or Handbook of Pharmaceutical Additives (compiled by Michael and Irene Ash, Gower Publishing Limited, Aldershot, England (1995)). On this basis, the compositions include, albeit not exclusively, solutions of the substances in association with one or more pharmaceutically acceptable vehicles or diluents, and may be contained in buffered solutions with a suitable pH and/or be iso-osmotic with physiological fluids. In this regard, reference can be made to U.S. Patent No. 5,843,456. As will also be appreciated by those skilled, administration of substances described herein may be by an inactive viral carrier. In one embodiment TCApP can be administered in a vehicle comprising saline and acetic acid.

(j) Kits

The reagents suitable for carrying out the methods of the invention may be packaged into convenient kits providing the necessary materials, packaged into suitable containers. Such kits may include all the reagents required to detect a nucleic acid molecule or peptide of the invention or conjugates of a nucleic acid molecule or peptide of the invention and another substance, such as a potential modulator of TCAP, and/or the detection of an indicator of TCAP activity, such as cAMP or cGMP, in a sample by means of the methods described herein, and optionally suitable supports useful in performing the methods of the invention.

In one embodiment of the invention, the kit includes primers which are capable of amplifying a nucleic acid molecule of the invention or a predetermined oligonucleotide fragment thereof, all the reagents required to

produce the amplified nucleic acid molecule or predetermined fragment thereof in the polymerase chain reaction, and means for assaying the amplified sequences. In one embodiment, the primers can amplify a nucleic acid encoding a TCAP protein, preferably the protein of SEQ.ID.NO.:.

The kit may also include restriction enzymes to digest the PCR products. In another embodiment of the invention the kit contains a nucleotide probe which hybridizes with a nucleic acid molecule of the invention, reagents required for hybridization of the nucleotide probe with the nucleic acid molecule, and directions for its use. In a further embodiment of the invention, the kit includes antibodies of the invention and reagents required for binding of the antibody to a TCAP peptide of the invention in a sample.

Before testing a sample in accordance with the methods described herein, the sample may be concentrated using techniques known in the art, such as centrifugation and filtration. For the hybridization and/or PCR-based methods described herein, nucleic acids may be extracted from cell extracts of the test sample using techniques known in the art.

The following non-limiting examples are illustrative of the present invention:

EXAMPLES

Example 1 Identification of Teneurin C-Terminal Associated Peptide (TCAP)

A. Identification of TCAP mRNA

Cloning of mRNA. A rainbow trout hypothalamic cDNA library was constructed as previously described (Barsyte et al., 1999) using a unidirectional vector (Unizap, Stratagene, La Jolla CA). A total of 600,000 clones were screened using a randomly labelled 305-bp hamster urocortin cDNA probe (Robinson et al., 1999)[SEQ. ID. NO 120 –5'-att cac cgccgc tcg gga tct gag cct gca ggc gag cgg cag cga cgg gaa gac ctt ccg ctg tcc atc gac ctc aca ttc cac ctg cta cgg acc ctg ctg gag atg gcc cgg aca cag agc caa cgc gag cga gca gag cag aac cga atc ata ctc aac gcg gtg ggc aag tga tcg gcc cgg tgt ggg acc cca aaa ggc tcg acc ctt tcc cct acc tac ccc ggg gct gaa gtc acg cga

ccg aag tcg gct tag tcc cgc ggt gca gcg cct ccc aga gtt acc ctg aac aat ccc gc-3'.] Primary, secondary and tertiary screens all utilized the same probe. The size of the clones, positive after the final screen, were determined by restriction analysis then sequenced using automated Big Dye methods.

Five positive clones were isolated from the rainbow trout hypothalamic library. Of these, one represented a partial sequence of a putative rainbow trout Ten-m3 homologue (Figure 1). The clone was 2986 bases long covering the translated portion of 769 bases]. SEQ. ID. NO. 1 shows a 756 base portion [SEQ. ID. NO. 2 thereof and a 3' untranslated region of 734 bases. The stop codon and translated portion were identified by alignment with the mouse (accession number AB025412)[SEQ. ID. NO: 132], human (accession number AK027474)[SEQ. ID. NO: 133] and zebrafish (accession number AB026976))[SEQ. ID. NO: 134], Ten M3 orthologues. Based on the human gene sequence (Locus Link ID# 10178) using Locus Link on the NICB server, the rainbow trout sequence included the terminal 6 exons of the gene. The final 3' exon encoded a 251 amino acid residue sequence [SEQ. ID. NO. 3] with a 40-41-residue carboxy-terminal sequence [SEQ. ID. NOS. 13 and 14, respectively] suggestive of a bioactive peptide. A putative amidation signal was indicated by the GKR amino acid motif immediately adjacent to the 40-41 residue carboxy terminal sequence and TAA stop codon. 40 residues upstream, a PC-7-like cleavage signal was present immediately followed by a glutamine suggesting that the putative free peptide would begin with a pyroglutamic acid. This cleavage site is not necessarily processed in the normal way and can create a 40 or 41 amino acid residue mature peptide (starting at 43 or 44 amino acid residues upstream from the stop codon).

B. Extraction of Free TCAP Peptide

Tissue Collection: Mouse brains (Mus musculus; n=10; 1.8g) were collected and stored at –80°C for one month, at which time they were removed and placed immediately into liquid nitrogen. Brain tissue was crushed using a mortar and pestle and powdered in the presence of liquid nitrogen.

Activation of C18 packing material: Bondpack® C18 bulk packing material (1g; 125Å; 37-55µm; Waters Corporation, Milford, MA, USA) was activated with 100% methanol (5ml), vortexed and left to stand (5min.). Excess methanol was removed. C18 was then washed in duplicate with PBS (5ml, pH 7.6). An additional PBS aliquot was added (5ml), vortexed and centrifuged (5000rpm; 5min); the supernatant was discarded.

Tissue Extraction: Acetonitrile (90%) and TFA (0.05%) were added to powdered brains in a 5:1 volume to weight ratio, mixed for 1 hr on an aliquot mixer rocker. The mixture was centrifuged (8000rpm X 20 min.); the supernatant was removed and saved. The remaining solids were back-extracted in acetonitrile (90%) and TFA (0.05%) in 40% of the solvent volume used in the initial extraction, vortexed and centrifuged as described previously. The supernatants were pooled and combined with activated C18 packing material, vortexed, mixed (1hr) and centrifuged (8000rpm X 10min). The supernatant was discarded while the pellet was subjected to three successive, independent acetonitrile extractions of 20%, 50% and 90% respectively. Acetonitrile (5ml) was added to the pellet, vortexed, mixed (20min) and centrifuged (6000rpm X 10min.). Resulting supernatant was saved and concentrated to 800µl on a vacuum concentrator (Brinkman Instruments) for HPLC analysis while the pellet was re-extracted in the same manner.

HPLC Purification of free TCAP in brain extracts

A Beckman model 126 HPLC System Gold (Beckman, Palo Alto, CA), attached to a UV detector module 168 and C18 column (3.5 um particle size; Waters Inc) was used to purify the TCAP peptide extracted from mouse brains (n=10).

A single injection (800ul) was applied to the column through a 1ml injection loop and carried to the column at a flow rate of 1ml/min using a dual solvent system (A: 0.05% trifluoroacetic acid (TFA); B: 80% acetonitrile, 0.05% TFA). Following an initial isocratic period of 10min, mobile phase B was increased from 10% to 60% over 75min, held isocratically for 5 min and

returned to 10% over 5 min. Fractions were collected (1ml/fraction), aliquoted (500ul) and concentrated to 50ul for analysis using mass spectrometry.

Example 2 Detection of the Cleaved TCAP in Cell and Tissue Extracts

HPLC as described in Example 1 can be used to detect TCAP. Mass Spectroscopy can also be used. Other detection methods can also be combined with HPLC, Mass Spectroscopy or used on their own, such as radio immunoassays, ELISAs, capillary electrophoresis, immunofluorescence confocal microscopy. Mass spectrometric methods identify molecules on the basis of a charged molecule's (ion) mass to charge ratio. A precise determination of the molecules mass is then determined allowing for identification of the molecule. Larger peptides can be sequenced by subsequent fragmentation of the peptide in a collision chamber. This causes preferential breaking of the peptide bonds. The amino acid and peptide fragments are identified by their mass to charge ratio. Radioimmunoassays or enzyme-linked immunosorbant assays (ELISA) utilize an antiserum specific for the molecule of interest. The molecule (TCAP) competes with a tagged structurally similar reference molecules to bind the antibody. The bound and unbound fractions are separated from each other and the quantity of remaining tagged TCAP is measured. This measurement is proportional to the amount of unlabeled TCAP present. Capillary electrophoresis can also be used to identify TCAP using an antibody reaction. In this method, the unbound component is separated from the bound component by migration in an electric field. Immunofluoresence confocal microscopy ulitizes a specific antibody bound to TCAP and a secondary antibody that binds to the primary antibody. The secondary antibody is effectively conjugated to an enzyme that catalyzes a fluorescent reaction upon introduction of the appropriate substrate. The amount of fluorescence is proportional to the amount of TCAP and is measured using digital image analysis.

Mass Spectrometry Detection of Peptide

Samples were dissolved in 5ul of 1:1 (vol/vol)Acetonitrile:water (plus 0.1%(vol/vol) formic acid). Typically, 2-3ul of each sample was loaded on a glass capillary probe tip and analyzed on a Micromass Q-TOF (hybrid quadrupole time of flight) mass spectrometer (Micromass, Manchester, UK). All spectra were acquired under nanospray, positive-ion mode. For MS measurements the quadrupole RF value was set at 0.5. The scanning region (m/z) was between 200-2000 with a scan time of 1 s and a dwell time of 0.1 s. The data was analyzed using MassLynx program (Micromass, Manchester, UK).

Example 3 Synthesis and Solubilization of Peptide

Rainbow trout TCAP-3 [SEQ. ID. NO: 13], wherein the terminal isoleucine (I) was amidated [to give SEQ. ID. NO. 15] was synthesized on an automated peptide synthesizer, Model Novayn Crystal (NovaBiochem, UK Ltd. Nottingham, UK) on PEG-PS resin using continuous flow Fmoc chemistry (Calbiochem-Novabiochem Group, San Diego, CA). Eight times excess diisopropyl ethy amine (Sigma Aldrich Canada Ltd) and four times excess Fmoc-amino acid activated with HATU (O-(7-azabenzotriazol-1-,3,3-tetramethyluronium hexfluorophosphate, Applied Biosystems, Foster City, CA) at a 1:1 (mole/mole) ratio were used during the coupling reaction. The reaction time was 1 hour. A solution of 20% piperidine (Sigma-Aldrich Canada Ltd) in N,N-dimethylformide (DMF; Caledon Laboratories Ltd, Canada was used for the deprotection step in the synthesis cycle. The DMF was purified in-house and used fresh each time as a solvent for the synthesis. The cleavage/deprotection of the final peptide was carried out with trifluoroacetic acid (TFA), thioanisole, 1,2 ethandithiol, m-cresole, triisopropylsilane, and bromotrimethyl silane (Sigma-Aldrich Canada Ltd) at a ratio of 40:10:5:1:1:5. Finally, it was desalted on a Sephadex G-10 column using aqueous 0.1% TFA solution and lyophilized. The peptide structure was confirmed by reverse-phase HPLC, amino acid analysis and atmospheric pressure ionization mass spectrometry. The HPLC and Mass spectrometry can be done as described in

Examples 1 and 2 herein. See above method. The same method was used to synthesize mouse TCAP-1.

The peptide was solubilized using a number of different methods, however, the best results were obtained using alpha cyclodextrin. Acetic acid (1ul) was added to dry TCAP at room temperature, vortexed and left to stand (30min). Alpha-cyclodextrin (company) was then added in a 4:1 volume to dry weight ratio (0.25ug/ul), vortexed, and concentrated to 10% of the original volume on an Eppendorf Vacufuge at 30 °C for 2h and room temperature for the remainder of the process. Distilled, de-ionized water and physiological saline were then added independently in a 1:1 and 3:1,volume to concentrated volume ratio respectively. This solution (0.5ug/ul) was vortexed and centrifuged (11,000 rpm; 3 min). The supernatant was aliquoted and stored at 4 °C. The same method was used to synthesize and solubilize other TCAPS including mouse TCAP-1.

Example 4 Peptide Sequence Relationships and Phylogeny

The rainbow trout Teneurin 3 exon including the TCAP portion shows a high degree of conservation among its orthologues in zebrafish, mouse, and humans (Figure 2). However the trout sequences also showed high sequence similarity with four mouse Teneurin protein paralogues designated as Teneurin 1 to 4 (Figure 3) and similarly four human paralogues were found in the sequence data base (Figure 4). All possess a high degree of similarity among members of the protein family. The Teneurin protein family represents a type II transmembrane protein where the carboxy terminus is displayed on the extracellular face of the plasma membrane (Figure 6 A and B). The TCAP portion represents only the C-terminal residues of the protein. The TCAP sequence is highly conserved across vertebrate species and even the Drosophila version possesses about 60% sequence identity (accession number AF008228) (Figure 7A and B).

Figure 5 illustrates the preTCAP nucleotide coding sequences for human, mouse, zebrafish and rainbow trout plus the stop codon. The coding

sequences for TCAP (40 and 41 amino acid residue sequences) can be easily determined from the figure.

A comparison of the conserved motifs within the primary structure of the TCAP and CRF families show a match (Figure 9). Conserved motifs of I/L-S-X-X (X)- L/V [SEQ. ID. NO: 129] at the amino terminus, L/V-L/I-X-V/aliphatic residue [SEQ. ID. NO: 130] in the middle and the motif N-I/A-H/basic residue-I/L/F-aliphatic residue [SEQ. ID. NO: 131] at the carboxy terminus. A more compelling gage of similarity, however, is shown by the secondary structure predictions (Figure 10). TCAP shows a highly similar polarity profile in comparison to others in the peptide superfamily. Hydrophobicity, using a Kyte-Doolittle plot shows a general similarity within the middle and carboxy terminal regions, but a more hydrophobic amino terminal region.

Although CRF and urocortin show high sequence similarity for each other and urocortin 2 and 3 show high similarity, the level of identity between these two paralogous lineages is only about 11%. The level of identity among TCAP members is about 60% (Figure 8). CRF and TCAP belong to a much larger peptide family that also includes the insect diuretic peptides (Figure 11). Key motifs, outlined in Figure 9 show alignment when the insect diuretic peptides are included.

Example 5 PCR Expression of Teneurin mRNA

The presence of the Teneurin protein in brain extracts and on cell lines were established using PCR. Primers utilized in this experiment were designed from 3'-ends of the published sequences for mouse Ten-M 1, 2, 3, and 4 [SEQ. ID. NOS: 4 – 7]. The TCAP-1 forward primer (25mer: 5'-ACGTCAGTGTTGATGGGAGGACTA-3')[SEQ. ID. NO: 121] is complementary to nucleotides 7938- 7962 of Teneurin 1. The Teneurin 1 reverse primer (27mer: 5'-CCTCCTGCCTATTTCACTCTGTCTCAT-3') [SEQ. ID. NO: 122] is specific for nucleotides 8262-8288 of Teneurin 1. The primers were predicted to generate a Ten-M1 PCR product of 351 bps. The Teneurin 2 forward primer (25mer: 5'-TCGAGGGCAAGGACACACACTACTT-3') [SEQ. ID. NO: 123] is complementary to nucleotides 7920-7944 of Teneurin 2. The Teneurin 2 reverse primer (26mer: AAGAACTGGATGTTGCTGCTACTGTC-

3') [SEQ. ID. NO: 124] is complementary to nucleotides 8354-8379 of Teneurin 2. The primers were predicted to get a Teneurin 2 PCR product of 460 bps. The Teneurin 3 forward primer (25mer: 5'-CAACAACGCCTTCTACCTGGAGAAC) [SEQ. ID. NO: 12]5 is complementary to nucleotides 7681-7705 of Teneurin 3. The Teneurin 3 reverse primer (21mer: 5'-TGTTGTTGGCACTGTCAGCCA-3') [SEQ. ID. NO: 126] is specific for nucleotides 8139-8159. The predicted PCR product for Teneurin 3 primers is 479 bps. The Teneurin 4 forward primer (23mer: 5'-TTTGCCTCCAGTGGTTCCATCTT-3') [SEQ. ID. NO: 127] is complementary to nucleotides 7868-7890 of Teneurin 4. The Teneurin 4 reverse primer (24mer: 5'-TGGATATTGTTGGCGCTGTCTGAC-3') [SEQ. ID. NO: 128] is complementary to nucleotides 8446-8469 of Teneurin 4. The primers were predicted to generate a Teneurin 4 PCR product of 602 bps.

The total RNA of Gn11 cells was isolated using RNeasy Mini Kit (Qiagen). First strand synthesis was performed by using First-Strand Beads (Amersham Pharmacia Biotech). Briefly, 2µg of total RNA was mixed with the first strand reaction beads (include buffer,dNTPs, murine reverse transcriptase, RNAguard, and RNase/DNase-free BSA) and 0.2µg random hexamer $pd(N)_6$ in a volume of 33µl. Extension was carried out for 60 minutes at 37°C.

The PCR for Teneurin 1,2,3,and 4 was performed respectively using 1µl cDNA with a final reaction volume of 50µl containing 0.2mM each dNTP, 5µl 10xbuffer, 1.5mM MgCl, 1ul Taq DNA polymerase, 0.2µM each Teneurin primer and 0.1µM each GAPDH primer (forward and reverse primers; The expected GAPDH DNA≈200bps). The initial denaturation was set over an interval of 3 min at 94°C. After 35 cycles of 1 min. at 94°C, 1 min. at 60°C, and 1 min. at 72°C, a 5 min. extension was performed at 72°C. The PCR products were examined by 1.5% agarose gel electrophoresis. The appropriate size DNAs of Teneurin 1, 2 and 4 were extracted from the gel using DNA extraction kit (MBI-Fermentas). The Teneurin 1, 2 and 4 DNAs recovered from the gel were subcloned by using the TOPO TA Cloning kit (Invitrogen Corporation). Briefly, the pCR® 2.1-TOPO plasmids with Teneurin 1, 2 or 4

DNA were transformed into chemically competent E. coli and cultured on LB agar plates and in liquid LB medium successively. The products were purified by using the Perfectprep Plasmid Midi Kit (Eppendorf). Positive results were selected by digesting the plasmids using the restriction endonuclease EcoRI and then by electrophoresis. The positive plasmids were sequenced commercially using T7 sequencing primer (AGTC Corp, Toronto, Canada).

Results

A positive amplification product was obtained from adult mouse cells for Teneurin 1, 2 and 4 using PCR (Figure 12). Similarily, the same products were obtained using mRNA extracted from the immortalized neuronal line, Gn11. A neuronal cell line isolated from the same tumour, NLT, showed expression of only Teneurin 2 and 4. However, a neuroblastoma cell line, Neuro2a appeared to express all four forms of the Teneurin gene family. The Neuro2a is the least differentiated of the cell lines used. A rat fibroblast cell line, TGR1, also showed the presence of paralogues 1, 2 and 4 (data not shown). The identity of the amplication signal was confirmed by sequence analysis. TCAP-1 primers generated a 351 bps sequence and showed 99.43% coincidence with Teneurin 1 DNA. TCAP-2 primers generated a 455 bps sequence and showed 99.56% coincidence with Teneurin 2 DNA. TCAP-4 primers generated a 602 bps sequence and showed 99.83% coincidence with Tenuerin 4 DNA. The TCAP 3 primers amplified a 306 bp sequence from mouse neuroblastoma Neuro2a cells. The amplified sequence possesses a 173-bp deletion upstream of the TCAP cleavage signal. This finding indicates that the TCAP-3 primers are specific, but that the Neuro2a cells appear to possess a variant of Teneurin 3 .

Example 6 Cell Proliferation Experiments

Several cell lines were utilized initially to establish a model system for which the TCAP could be evaluated. Initially the mouse neuroblastoma cell line, Neuro2a, the human breast cancer cell line MCF-7, mouse GnRH-secreting immortalized neuron lines NLT and Gn11 COS-7 cells, and the rat fibroblast cell line TGR1. Preliminary studies indicated that the cells were

responsive to the effects of TCAP Rainbow Trout TCAP-3, SEQ.ID. NO:.13: amidated [SEQ. ID. NO. 15], in that the cells showed a decrease in cell proliferation (data not shown). The studies were performed essentially in accordance with the cell proliferation studies below. Gn11 and TGR1 cells were selected to be used for further studies.

Pharmacological Test of TCAP on fibroblast Cell Lines TGR1 and HO16.4c: 2 plates containing $3x10^4$ TGR1 cells/well and 2 plates containing $3\leftrightarrow10^4$ HO16.4c cells/well in full-serum medium were prepared for testing. Each 6-wells in the plate was designed as a testing group. 24 hours later, aliquots (20 µl) of drugs) were added in a 12-hours interval after changing the medium using fresh full-serum DMEM. The cells were observed through a microscope per 4-hours. The numbers of the two cell lines were found significantly lower in TCAP groups at 48-hrs and 72-hrs stages. Cells were counted at 48 hours and 72 hours after being treated. Two plates containing $3 x10^4$ Gn11 cells/well in full-serum medium were prepared for testing. Each 6-wells in the plate was designed as a testing group. 24 hours later, aliquots (20 µl) of drugs (vehicle:saline+acetic acid; 10^{-6} M TCAP-3) were added in a 12-hours interval after changing the medium using fresh full-serum DMEM. The cells were observed through a microscope per 4-hours. Cells were counted at 48 hours and 72 hours after being treated.

A concentration of 10^{-6} M of TCAP administered at 0, 12 24 and 36 hours decreased the proliferation of a mouse neuronal cell line (Gn11) (Figure 13A – 48 hrs and 13B – 72 hrs), a rat fibroblast cell line(TGR1) by 50-60% at 48 hours (Figure 14)and a HO16.4c cells at 48 hours relative to the vehicle treated cells (Figure 15).

The ability of TCAP to inhibit cell proliferation in the above-noted cell lines, indicates that the peptide would be useful in the regulations of cell proliferation and associated medical conditions such as in the treatment of cancer TCAP could be used to arrest tumour growth and inhibit metastasis. In a preferred embodiment, TCAP could be used in the treatment of neuronal tumors.

Example 7 Cyclic Nucleotide Experiments

I. A. cAMP and cGMP assays

Approximately 10^6 Gn11 cells were treated with 20 uL of 10^{-9}, 10^{-8}, or 10^{-7} or 10^{-6}M TCAP-1 or TCAP-3 and incubated at 37 C for 10 minutes. Medium and peptide was removed and the cells were lysed using 350 uL of a 0.1 M HCL 0.1 % Triton X-100 solution. Using the same concentrated HCl and Triton X-100 solution and a provided standard concentrate, five standard solutions were made up with concentrations of 200, 50, 12.5, 3.12 and 0.78 pmol/ml. All reactions were done in triplicates. Wells were set up for blanks, non-specific binding, total activity(TA), zero binding, five standards, and 12 samples. Using a 96-well IgG coated plate, 50 uL of neutralizing reagent were pipetted into each well except the blanks. 150 uL of the 0.1 M HCL/0.1 % Triton solution was pipetted into the NSB wells and 100 uL of this solution was pipetted into the zero binding wells. 100 uL of the standards and 100 uL of the samples were pipetted into their respective wells. 50 uL of conjugate were pipetted into each well except the TA and the blank wells. 50 uL of the cAMP antibody were pipetted into each well except the TA, blank and NSB wells. The plate was allowed to shake overnight. The following morning, the wells were rinsed three times with a 10 times diluted wash buffer solution. 50 uL of conjugate was added to the TA wells and 200 uL of p-Npp substrate was added to each well. The plate was covered again and incubated at room temp for one hour. At this point, 50 uL of stop solution was added to all wells and the absorbance was read at 405 nm using a Spectramax spectrophotometer. Three levels of controls were utlized: A blank tube which provides a measure of any reactivity between p-Npp substrate and IgG coated wells.;TA: measure of activity of alkaline phosphotase in conjugate, if any; NSB: measure of binding of conjugate to plate or to antibody; Bo: measure of binding conjugate to antibody (no sample and conjugate competition).

B. Results

In the first set of experiments, Gn11 cells were treated with 10^{-6} M of rtTCAP-3 SEQ. ID. NO:13, amidated [SEQ. ID. NO: 15], see above, rat

urocortin or the vehicle, as above (Figure 16A). TCAP reduced cAMP accumulation in these cells to 58.9 ± 4.8 % of the vehicle- treated cells ($p<0.01$). Urocortin induced a non-significant decrease of 89.2 ± 6.3% of the control cells. In cGMP accumulation experiments, TCAP reduced cGMP accumulation to 38.5 ± 8.8 % of the control cells ($p<0.01$) whereas urocortin caused a decrease to 50.0 ± 8.5% of the control cells. (Figure 16B)

II. A. cAMP Assays

Gn11 cells were treated when the confluence reached 70%. The cells were treated with 10^{-9}, 10^{-8} or 10^{-7}M TCAP, urocortin and vehicle, separately, and incubated in incubator at 37 °C. (Details below) Medium was removed and the cells were washed by PBS one time, and then were lysed using 600 uL of 0.1 M HCL solution. After freezing/thawing 3 times, the samples were transferred into microcentrifuge tubes. At the same time, squeezed the cells by 3 ml syringe and 22G needle 20 times. Centrifuge 4000rpm ×5 min, the supernatant of each sample was aspirated and kept in the –20 °C freezer until the cAMP or cGMP assay was carried on. Using the same concentrated HCl and a provided standard concentrate, five standard solutions were made up with concentrations of 200, 50, 12.5, 3.12 and 0.78 pmol/ml. All reactions were done in duplicates. Wells were set up for blanks, non-specific binding (NSB), total activity (TA), zero binding (B0), five standards, and all samples. Using a 96-well IgG coated plate, 50 uL of neutralizing reagent were pipetted into each well except the blanks and TA. 150 uL of the 0.1 M HCL was pipetted into the NSB wells and 100 uL of this solution was pipetted into the zero binding wells. 100 uL of the standards and 100 uL of the samples were pipetted into their respective wells. 50 uL of conjugate were pipetted into each well except the TA and the blank wells. 50 uL of the cAMP antibody were pipetted into each well except the TA, blank and NSB wells. The plate was allowed to shake overnight (18h) at 200 rpm at 4 °C. The next day, the wells were rinsed three times with a 10 times diluted wash buffer solution. After each well was dried thoroughly, 5 uL of conjugate was added to the TA wells and 200 uL of p-Npp substrate was added to each well. The plate was covered again and incubated at room temp for one hour without shaking. At

this point, 50 uL of stop solution was added to all wells and the absorbance was read at 405 nm and 580nm using a Spectramax spectrophotometer. The data of 580 nm were provided the background of each well, which were substracted from the data of 405 nm.

B. Results

10^{-8} M TCAP induced a significant increase in cAMP accumulation at 15 minutes after introduction of the peptide and fell to normal limits within 30 minutes of treatment (Figure 17A). Urocortin was used for the purpose of a positive control. Figure 17B illustrates cAMP levels in Gn11 cells in the presence of 10^{-4} M 3-isobutyl-1 methyl xanthine (IBMX), a phosphodiesterase inhibitor used to boost cAMP induced by treatment of 10^{-8} MTCAP or urocortin. Figure 17C is a bar graph illustrating cAMP accumulation over 30 minutes in Gn11 cells by administration of various concentrations of TCAP or Urocortin in the presence of IBMX. Figure 17D is a bar graph illustrating inhibition of 10^{-6} M forskolin- stimulated cAMP by 10^{-8} MTCAP or urocortin.

Example 8 Behavioural Studies

A. Brain Stimulation Reward Behaviour Experiments

Rats can be trained to bar press for electrical stimulation of the lateral hypothalamus which activates cholinergic nuclei of the pontine tegmentum and their projections to dopaminergic paths of the forebrain. Once reliable baseline rates of bar pressing have been established for a given current, the consequences of various drugs for the activity of this cholinergic dopaminergic system can be assessed by making injections of substances intracranially and then observing their effects on rates of self stimulating behaviour. TCAP-3 SEQ. ID. NO: 13, amidated, [SEQ. ID. NO. 15] see above, at a concentrations of 1 nM prepared in physiological saline was injected by canulae into the laterodorsal tegmental nucleus through guide cannulae. The rate of bar pressing was compared to the vehicle treated rats.

B. Results

A robust inhibition of self-reward stimulus occurred when TCAP at 1 nM (4.2 pg/ul) was injected into the caudal midbrain of rats (Figure 18). In both forebrain (lateral ventricle) and midbrain injections the effect was reversible with the rats behaviour returning to normal limits after about 60 minutes.

Example 9 Preliminary *in situ* Hybridization Results

The first in situ hybridization data indicate that the Teneurin I gene (TCAP-1) is highly expressed in adult rat brain. The regions of greatest expression occur in the lateral septum, bed nucleus of the stria terminalis ventral medial nucleus of the hypothalamus and ventral premammalary nucleus. Lesser expression occurs in the hippocampus and amygdala. This expression pattern is consistent with peptides regulating the stress response (see above) in emotional and mood disorders. These data indicate that TCAP plays a primary role in stress and anxiety regulation rather than one of neurogenesis and neurodegeneration. The Teneurin 4 (TCAP-4) expression also occurs in the adult brain but Teneurin 1 is stronger.

A. Methods

The methods were performed as previously described (Simmons et al., 1989; Ericsson et al., 1995) using ^{35}S-labelled antisense and sense (control) probes higher high stringency conditions (50% formamide with final washes at 0.2 SSC at 60 C). The ^{35}S-labelled cRNA probes were generated from 350 bp cDNA of exon 33 including the TCAP portion by in vitro transcription with the appropriate polymerases (T3 for antisense and T7 for sense).

B. Results

Results are shown in Figure 20. On the left column is the expression of TCAP-1 mRNA using the antisense probe, and on the right column, the sense probe. A-B. central nucleus of the amygdala (CeA); C-D. bed nucleus of the stria terminalis, medial (BSTM); E-F: premammilary ventral nucleus (PMV).

Abbreviations: 3V, third ventricle; fx, fornix; ic, internal capsule; LV, lateral ventricle; MeA, medial nucleus of the amygdala; opt, optic tract; st, stria terminalis. Bars = 300 µm (A-B) and 500 µm (C-F)

The in situ hybridization data indicate that the TCAP-1 gene is highly expressed in adult rat brain. The expression of the C-terminal teneurin-1 exon including the TCAP-1 region was restricted to hypothalamic and limbic regions (Fig 20 A-F). The regions of greatest expression occur in the lateral septum, bed nucleus of the stria terminalis ventral medial nucleus of the hypothalamus and ventral premammalary nucleus. Lesser expression occurs in the hippocampus and amygdala. This distribution is consistent with TCAP playing a modulatory role with emotionality, anxiety and motivation. The presence of TCAP-1 expression in the ventral premammillary nucleus is of particular interest as there are no known CRF receptors found in this region (Li et al., 2002). There was no evidence that the TCAP containing exon was expressed in regions associated with neurogenesis, such as the olfactory lobes or subependymal layers of the lateral ventricles. Despite the previous recognition of the teneurin proteins, their expression in adult brain has never been examined. However, teneurin 1 and 4 expression has been observed in the diencephalon of developing mouse, chick and zebrafish brain (Rubin et al., 1999; Ben-Zur et al, 2000; Mieda et al., 1999).

These data support the hypothesis that TCAP primary role is one of stress and anxiety regulation.

Example 10 Chronic TCAP study: The Role of TCAP In Modulating The Stress Response

A. Method

1. Wistar Rats were tested in acoustic startle for baseline response (1 hour test consisting of 60 acoustic startle stimuli, 120 dB, 60 sec inter-stimulus interval), and divided into matched groups to receive either TCAP-1 (10 nmol of mouse TCAP-1, amidated [SEQ. ID. NO. 40] in 3µl vehicle intra-cerebroventricularly) or Vehicle (e.g. saline and acetic acid).

2. Two days later, rats were tested in acoustic startle, 25 stimuli baseline (120 dB, 60 sec inter-stimulus interval), then injected ICV with 10 nmol TCAP-1 or Vehicle, then acute response was measured for 1h (60 stimuli, 120 dB, 60 sec inter-stimulus interval).
3. 25 days later, rats were given either TCAP-1 (10 nmol in 3µl or vehicle (3µl once per day for 5 consecutive days ICV.
4. Rats were left alone for 10 days.
5. On the 10th day, rats were tested for acoustic startle response without TCAP-1.

On the 11th day, rats were re-tested for startle response, again without TCAP-1, for 60 minutes (60 stimuli, 60 sec inter-stimulus interval, 120 dB). Re-tested in startle 13th and 28th days. The vehicle is the mixture of saline and acetic acid into which TCAP-1 was dissolved . When referring to vehicle, this refers to the solution without the addition of TCAP-1.

B. Results

Results are shown in Figure 21 for the 0, 10 and 12 days after the 5 consecutive day ICV of Vehicle (21A) or TCAP-1(21B). Startle responses for animals in the chronic study are shown in Figure 22. The average startle response for the two groups (TCAP-1 and Vehicle) on Day 1, before chronic TCAP treatment is shown in Figure 22A. Figure 22B shows the average startle response for TCAP and vehicle groups over the 60 trials in the session on the 10th day after chronic TCAP treatment. Figure 22C shows the mean baseline startle responses for all animals for TCAP and vehicle groups averaged across all 60 trials.

Example 11 Acute TCAP Study Acoustic Startle Measurements

A. Method

Male Wistar rats (250-275 g), were surgically implanted with cannulae (23 gauge) bilaterally into the basolateral nuclei of the amydala (AP –2.8, ML +/- 5.0, DV – 7.2 mm, from bregma). One week later, the animals were habituated to the acoustic startle reflex (ASR) chambers (MED Associates,

grid rod cage measuring 7.5" x 3.6" x 4.2"), consisting of 25 trials of 120 dB stimuli presented randomly with an inter-stimulus interval of 55-65 seconds, duration of 30 msecs and frequency of 5000Hz. The same stimulus conditions were used for test days, which consisted of a 25 trial baseline, injection with mouse TCAP-1 (with amidation signal)[SEQ. ID. NO. 40] or vehicle (0.25 µl/side, flow rate 0.5µl/min), and testing for a further 60 trials post-drug. Each rat received vehicle treatment on the first test day then TCAP-1 (e.g. mouse TCAP-1) in a random and counter balanced fashion in subsequent test days, spaced 48h apart. On the final test day, all rats again received vehicle treatment. Following histological analysis of cannulae placements, the data of eight rats was retained for statistical analysis.

From the data, rats were divided into high and low anxiety groups depending upon their treatment/baseline ratio for the vehicle. Animals that scored less than one were considered low anxiety, those scoring more than one were considered high anxiety. There were four animals in each anxiety group.

Results are shown in Figures 23 and 24. Figure 23 is a bar graph illustrating the mean treatment/baseline value for both groups for all concentrations of mouse TCAP-1. A repeated measures ANOVA indicated that the level of significant differences between the two anxiety groups was P=0.0078. After TCAP-1 treatment the treatment/baseline ratio of low anxiety was similar to the initial high anxiety value and vice versa. A vehicle injection was performed at the end of the study to show that the effect was due to the TCAP-1 and not to the experience of injection. TCAP 1 concentrations were 3, 30, 300 pmoles. A summary of the effect of amygdala-injected TCAP-1 is illustrated in Figure 24. It was shown that the effect by TCAP-1 on startle response is inversely proportional to the baseline startle response. As such TCAP-1 can be used to normalize startle behaviour or stress response.

Discussion

Regardless of the mechanism the synthetic TCAP peptide is potent, in vivo at eliciting a behavioural response in rats. Given the strong expression of

TCAP in hypothalamic and limbic regions, the synthetic mouse TCAP-1 peptide with amidation signal was micro injected into the basolateral amygdala to determine effects on acoustic startle in rats. Animals possessing a high treatment-to-baseline ratio (>1) showed a significant ($p<0.05$) decrease in startle magnitude, whereas animals with a low treatment-to-baseline ratio (<1) showed a significant (p,0.05) and does dependent increase in startle magnitude (Figure 23). These data indicate that TCAP-1 acts to modulate the effect on startle responses depending on baseline reactivity of the particular animal and can normalize the behaviour associated with acoustic startle. Other neuropeptides that have been demonstrated to increase acoustic startle are CRF (Liang et al., 1992), CCK (Frankland et al., 1997) and SP (Krase et al., 1994/1999). The acoustic startle paradigm is a well-known and extensively used paradigm for assessing the anxiogenic or anxiolytic effects of drugs. This is an ideal paradigm for testing a novel compound since the startle reflex does not involve locomotion, learning, memory, or motivated behaviour of any kind, which could possibly confound the interpretation of the results.

The data presented indicate that TCAP represent a new family of neuropeptides associated with the regulation of anxiety by regulating neuronal function in key regions of the forebrain and limbic system. Previous studies have also suggested a role of the teneurin genes with neural regulation. Human Ten-M1 maps to position Xq25 of the X chromosome (Ben-Zur et al., 1999). This is a region associated with X-linked mental retardation syndromes (Minet et al.,1999). The conditions mapped to this site are characterized by severe mental retardation and may include motor sensory neuropathy, deafness and sometimes seizures and impaired vision.

The regulation of TCAP represent a new target to understand the aetiology of neurological dysfunction and psychiatric illness. The example shows that TCAP can be used in the treatment of stress-reated disorders and in other neuropathological conditions.

Example 12 Activity of TCAP on immortalized neurons.

A. In vitro assays

Gn11 immortalized neurons were cultured as previously reported (Tellam et al., 1998) Direct cAMP measurements were performed with the nonacetylated version of a commercial kit (Assay Designs, Ann Arbor, MI). After starved by serum-free DMEM for 1 hr and replaced with fresh DMEM without serum, cells were treated for 15min with TCAP, urocortin or vehicle ± CRFR1 antagonist PD171729 in the continued presence of forskolin (1 _M) and IBMX (100 _M. Protein assays: Total protein was determined using the BCA protein assay method (Pierce Co). MTT Assays: Gn11 cells were seeded into 96-well plates and cultured in full serum DMEM until the cells were 30% confluent. Vehicle, 1nM, 10nM or 100nM TCAP-1 were added into each group (n=8). (Figure 25A) The MTT assay (Sigma Chemicals) was performed at 0, 6, 12, 24 and 48 hours. Flow Cytometry: DNA content of the Gn11 cells was quantified by staining with propridium iodide and analyzed on a FACSCAN flow cytometer (Beckman Instruments).

B. Results

Mouse TCAP-1 induced a dose-dependent change in cAMP accumulation in mouse immortalized neurons after 15 minutes. A 1 nM dose increased ($p<0.05$) cAMP levels 45% over the vehicle-treated cells. In contrast, 100 nM TCAP-1 decreased ($p<0.05$) cAMP accumulation 40% from control cells (Figure 25A). However, co-treatment with the specific CRF type 1 receptor antagonist, PD171729 failed to completely abolish TCAP's effects at cAMP accumulation. In contrast, the same concentration of antagonist induced a complete inhibition ($p<0.01$) of urocortin-stimulated cAMP accumulation in these cells (Figure 25B). We have previously established that these cells possess a CRF-R1 receptor (Tellam et al., 1998) but not an R2 receptor (data not shown). Concentrations of 1, 10 and 100 nM of TCAP-1 induced a significant increase in total protein concentration after 120 minutes in the same cells (Figure 25C). Mouse TCAP-1 treatment of these cells also induced a dose-dependent effect on cell metabolism. Cellular activity as

indicated by mitochondrial activity (MTT assay) showed a significant ($p<0.05$) increase in activity at 1 nM concentration, but a decrease at 100 nM concentrations (Figure 25D). Similarly, 1 nM TCAP reduced ($p<0.05$) the incidence of G1 phase after 24 hours whereas a 100 nM dose increased ($p<0.05$) G1 phase as determined by DNA content analysis.

As such α-helical CRF(9-41) antagonist can modulate TCAP stress response modulating activity.

Example 13 Proteomic Profiling and MicroArray Studies

To determine the effect of TCAP and to develop a cell model system for screening TCAP modulators, diagnostic and conditions related to TCAP and methods of medical treatment, TCAP responsive cell lines were subject to proteomic profiling and microarray analysis. This was done using a non-tumorgenic-derived immortalized murine hypothalamic cell line, N38, which has the marker profile shown in **Table 1**. The effect of TCAP on other immortalized cell lines can be preformed by adapting the method noted below.

A. TCAP Responsive Immortalized Hypothalamic Cell Lines

The TCAP responsive immortalized cell lines used were prepared by Denise Belsham, University of Toronto, by preparing a culture of embryonic hypothalamic cells; infecting said culture with a retrovirus encoding a viral oncogene, large T Antigen, operably linked to a promoter and a selectable marker; isolating transfected cells from non-transfected cells to obtain a culture of immortalized hypothalamic cells; subcloning said immortalized cells into sub-cloned populations; and screening said subcloned populations for expression of specific neuronal markers; and selecting and further cloning a specific population. The immortalized cell lines can then be screened for TCAP responsiveness.

TCAP responsiveness was screened by measuring the functional cAMP response of the immortalized subclones to TCAP . The results are shown in Figure 26. N-15-1, #7 (N7), N-18-1, #11 (N22), and N-15-14, #29

(N29) were analyzed for the cAMP response to peptide stimulation. The subclones were split into 24 well plates. Cells were starved for 1 h in DMEM without FBS, then medium was replaced with 0.5 ml fresh DMEM (without FBS) with the compounds as indicated. In Figure 26, neurons were exposed to 10^{-7} M (100 nM) TCAP peptide. All peptides were diluted in DMEM containing IBMX (100 µM). After a 15 min incubation at 37 °C, 1 ml of ice-cold ethanol was added to each well. Cells were scraped from the plate and kept at -20 °C until the amounts of intracellular cAMP were determined in triplicate by RIA (Biotechnologies Inc., Stoughton, MA) according to the manufacturer's instructions.

B. Proteomic Profiling Using TCAP 3

NPY17 (N38) immortalized neurons were treated with 100 nM TCAP-3 and subjected to proteomic profiling. In this procedure, the nuclei of cells are isolated and the proteins extracted. This method provides an indication of proteins that are up or down regulated by a given treatment. The proteomic profile indicated that the majority of proteins up-regulated were associated with cell cycle, metabolism and the stress response. A. number of cytoskeletal proteins were also upregulated. This observation is of particular importance as many antidepressants have been shown to increase spine density and arborization of neuronal processes. Such events are regulated by cytoskeletal proteins.

Proteomic profiling
Up regulated at 12 hours

Protein Processing
Parvulin; protein chaperone

Transcriptional Regulation
Npw28 binding protein
Staufen; mRNA targetting
histone acetylmethyl transferase helicase

Cell Growth, Cycle and Proliferation
MIDA1; cell growth regulator
Smad 5; TGFbeta signalling
STE20-like kinase; apoptosis
Kp78, wnt pathway activation
Integrin linked kinase 1, wnt pathway p53 target protein,
tumor suppressor IGFBP, growth regulation
esp1, cell division
sepiapterin reductase
TGFbeta Bp1, growth regulation Rad23, uv repair protein

Extracellular Matrix
protocadherin gammaB5
talin

Cytoskeleton
alpha actinin4
CLP36, actinin4 interaction

Cell Signalling
PKC iota

B. MicroArray Studies

I. Method

RNA isolation

Total RNA (TRNA) was isolated from 3 independent treated and untreated N38 hypothalamic cell cultures, pooled (to reduce the noise), utilizing Trizol Reagent (GIBCO/BRL) following the manufacturer's protocol. The quality of total RNA was assessed using an Agilent 2100 Bioanalyzer (version A.02.01S1232, Agilent Technologies). Only RNA with the OD ratio of 1.99-2.0 at 260/280 was used.

Oligonucleotide Arrays (Hybridization, Staining, and Scanning)

Hybridizations were performed on the Mouse MU74Av2 GeneChip Set (Affymetrix, Santa Clara, CA). Samples were prepared for hybridization according to Affymetrix instructions. Briefly, a primer encoding the T7 RNA polymerase promoter linked to oligo-dT_{17} was used to prime double-stranded cDNA synthesis from each mRNA sample using Superscript II RNase H$^-$ reverse transcriptase (Life Technologies, Rockville, MD). Each purified (Qiaquick kit, Qiagen) double-stranded cDNA was in vitro transcribed using T7 RNA polymerase (T7 kit; Enzo), incorporating biotin-UTP and biotin-CTP (Enzo Biochemicals, New York, NY) into the cRNAs, followed by purification using RNEasy (Qiagen) and quantitated by measuring absorption at 260 nm/280 nm. Samples were fragmented and hybridized to the Chip for 16 h at 45°C and scanned (GeneArray scanner, Affymetrix). MicroArray Suite Version 5 (MASv5; Affymetrix) was used to scale intensities across the Genechips to 150 fluorescence units, and to determine expression values for each gene on the chip. The expression value for each gene was determined by calculating the average of differences (perfect match intensity minus mismatch intensity) of the probe pairs in use for the gene.

Data Analysis

Gene analysis software: Data analysis was performed using two independent softwares, GeneChip and GeneSpring. To identify differentially expressed transcripts, pairwise comparison analyses were carried out with MicroArray Suite Version 5 MicroArray Suite Version 5 (MASv5; Affymetrix). This approach, which is based on the Mann-Whitney pairwise comparison test, allows the ranking of results by concordance, as well as the calculation of significance (*P* value) of each identified change in gene expression. Statistically significant genes ($P<0.05$) were selected for further analysis. Moreover, statistically significant changes in mean expression values were determined by importing the data from MASv5 into GeneSpring 5 (Silicon Genetics, Redwood City, CA). A stepwise process was followed, first with normalizations. A per-chip followed by a per-gene normalization in order to facilitate direct comparison of biological differences. Next, a second method of filter using Affymetrix data and p value with cut-off of $P<0.005$ generated 4,841 genes which were used for subsequent analysis utilizing Hierarchical Clustering, k-means, Self Organization Map (SOM) utilizing GeneSpring 5.0.

II. Results

Further, to demonstrate that the cell lines can be used as a model for studying TCAP responsiveness, modulation, and in screening for TCAP modulators, microarray studies were performed on 1 nM TCAP-1 [SEQ ID NO 5 plus amidation signal GRR at C-terminus] treated N38 hypothalmic cells, which do not possess either CRF receptor subtype (Table 4). RNAs isolated from treated and untreated cells were analyzed on oligonucleotide arrays representing 12,884 mouse genes (Affymetrix, http://www.affymetrix.com). Standard filtering ($p< 0.005$) and hierarchical clustering algorithm (average linage method: GeneSpring software – Silicon Genetics) identified significant changes in the expression of 4, 841/12,885 genes with 166 genes showing 1.5 fold down-regulation and 35 genes up-regulation in the TCAP-1-treated cells compared to the untreated cells. At 16 hours post-treatment, a significant decrease occurred among several genes, notably, GAS5, SDPR and CD95 that have been associated with growth arrest or apoptotic events

(45-47). In contrast, upregulated genes including MK167, MOP3 and GDAP10 have been associated with cell proliferation and cell cycle modulation (48-50). A G-protein coupled receptor-related signal transduction pathway is indicated by the regulation of genes, CREM, AKAP8, AKAP95 and PDE6A. Downstream effectors of RAS such as EFK1 and RGL were also down regulated. Downregulation of the A kinase anchoring protein AKAP95 but upregulation of AKAP8 suggests that TCAP may act, in part, by changing the targeting pattern of PKA (51). The upregulation in inducible nitric oxide (INOS), a intracellular voltage-gated chloride channel (CLCN3) and the serotonin transporter (SLC6A4) may reflect the down stream actions of cAMP-mediated signal cascade and indicates the potential for TCAP to be involved in neuronal signaling systems. A role in interneuron communication by TCAP is also indicated by the modulation of genes associated with the regulation of vesicle trafficking. Thus, the TCAP responsive cell lines can be used to screen for modulators of neuronal function that affect growth, differentiation and communication.

SUMMARY OF EXPERIMENTAL RESULTS

The teneurin c-terminal associated peptide (TCAP) represents the terminal 40 to 41 residues on all four of the known teneurin (Ten M) proteins. On all four of the teneurins, TCAP shows the greatest sequence homology among the entire exon suggesting that it is under the most stringent physiological constraints of the protein. TCAP is a potent inhibitor of neuronal and fibroblast growth possibly by arresting cell cycle. When injected into rat brain it increased the startle reflex and decreased self-administered reward behaviour and was shown to modulate the stress response. These data indicates that TCAP represents a novel neurohormonal system associated with neuronal growth and development.

The finding of a TCAP-like peptide on the carboxy terminus of a type II transmembrane protein is unusual. Assuming that the protein is only expressed on the extracellular face of the cell, then it is likely that the peptide acts in a paracrine manner to regulate the surrounding cells. All Ten M

proteins possess a basic residue in positions –1 and –8 upstream from the putative cleavage site from the peptide. Such a basic residue arrangement is recognized by the prohormone convertase 7 (PC7) family of proteases (Saideh and Chretien, 1997), for the processing of peptide prohormones. Assuming this to be the case, then the requisite PC7-like protein would need to be expressed also on the extracellular face of the cell, or perhaps on the extracellular face of an adjacent cell. Alternatively, the protease may be secreted and act in a more mobile fashion. In any case, the release of the cleaved peptide would unlikely to occur in the bolus seen by vesicular release. It is also conceivable that the Ten-M protein is expressed in vesicles of the regulated pathway where intravesicular proteases could cleave the peptide before exocytosis. However, the synthetic peptide shows a strong tendency to aggregate and precipitate at concentrations higher than 2 ug/ul. This is likely due to the high number (15) of leucines, isoleucines, valine, tyrosines and phenylalanine within the peptide. Peptides that have high vesicular concentrations such as the urocortin-like peptide, sauvagine, found in the skin of a neotropical frog, *Phyllomedusa sauvagei,* tend to have a low proportion of hydrophobic residues (Pallai et al., 1983). Thus this physical characteristic of the TCAP peptide supports its preferential release from the cleavage from the extracellular face of the plasma membrane.

The TCAP portion of the Ten-M proteins appears to be the most highly conserved of the terminal exon of the protein. Such high levels of conservation occur when there are many physiological, biochemical constraints acting upon the sequence to inhibit change. Such resistance to change could result from essential interactions with processing or degrading enzymes, receptors, and/or transport proteins. The level of conservation of 90% between the paralogues in vertebrates is high in comparison to the CRF group of peptides to which TCAP appears to be most closely related.

In any case, a number of other bioactive peptides are initially expressed and processed in the same manner as TCAP. Other bioactive peptides such as tumor necrosis factor (TNF) (Utsumi et al., 1995), Apo-2 ligand (Pitti et al., 1996) and fractalkine (Garton et al., 2001) are processed in

this manner. These peptides are directed outward at the end of the C-terminus on the extracellular face. Peptides processed and expressed in this manner have the potential for a variety of endocrine or juxtacrine roles. For example they may act as an adhesion molecule for cells displaying the appropriate receptor. Such actions could be particularly important during the migration of neurons in the developing brain, allowing neurons to be directed to a specific target. Alternatively, the peptide may be cleaved via a membrane-bound or extracellular matrix-associated protease to act as a paracrine/autocrine factor to modulate the actions of surrounding cells. Such a mechanism would be important for cells to protect against low oxygen stresses which occur in ischaemia. All three cytokines appear to be processed by a tumor necrosis factor alpha converting enzyme (TACE, ADAM17). This enzyme is also capable of cleaving the cell-surface ectodomain of the amyloid-beta precursor protein (Skovronsky et al. 2001), thus decreasing the generation of amyloid beta suggesting it may have a role in the aetiology of Alzheimer's disease.

The TCAP peptide appears to regulate several physiological events. In a mouse neuronal cell line, Gn11, and a rat fibroblast cell line, TGR1, treatment of TCAP at concentrations of 10^{-9} to 10^{-6} M could inhibit proliferation in a dose-dependent manner where maximal inhibition occurs at about 60%. There was no evidence of apoptosis or necrosis of the cells and morphology did not differ between treated and untreated cells.

This stress-related studies indicate an ability of the TCAP peptide to inhibit the damage done by environmental stresses on cells that would occur during periods of ischaemia or perhaps various neurodegenerative diseases. Given the decrease of proliferation rate seen in unstressed cells and the apparent increase in stressed cells suggests that TCAP may be acting in part to reduce the metabolic activity of the cell. Other related peptides have a similar effect. For example, urocortin can prevent cell death in primary cardiac myocyte cultures by stimulating the p42/p44 mitogen-activated protein (MAP) kinase pathway (Latchman, 2001). Under stressful conditions such as heat shock (Okosi et al., 1998) or ischaemia (Brar et al., 1999), urocortin mRNA is

upregulated in cultured cardiac cells, and is also secreted into the medium (Brar et al., 1999), suggesting that it too, is acting in a paracrine fashion to regulate cell metabolism. This effect is much greater by urocortin than CRF. This is of particular interest given that the urocortin paralogues of the CRF family appear to represent evolutionarily older sequences than CRF (Lovejoy and Balment, 1999). Such paracrine actions on cell metabolism may be then one of the initial and critical functions of the ancestor gene that gave rise to both the TCAP and CRF/urocortin/diuretic group of peptides.

The data obtained so far can be used to delineate a tentative model for the mechanism for TCAP (Figure 19). Initially, a stressor, such as changes in pH, temperature, or O_2 levels, or alternatively, a stress-induced ligand triggers an up-regulation of the Ten-M protein. Such stressors likely act through a number of signal transduction pathways including adenylate cyclase and guanylate cyclase. It is conceivable that the stressor also up-regulates the Ten-M cleaving enzyme such as TACE or PC7. The TCAP ligand is then cleaved from its protein and is free to act in an autocrine and paracrine manner. It binds to a G-protein coupled receptor that subsequently interacts with a G-inhibitory protein. This inhibits cAMP and cGMP production to inhibit activation of the cell. In a dividing neuron this would act to inhibit proliferation or migration, and in an mature non-dividing neuron could manifest as a reduction of synaptic output thereby inhibiting the neurological response of an activated nucleus of cells in the brain.

While the present invention has been described with reference to what are presently considered to be the preferred examples, it is to be understood that the invention is not limited to the disclosed examples. To the contrary, the invention is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

Table 1: Cell Lines Screening

MARKER	N-7	N-22	N-29	N-38			
T antigen	+	+	+	+			
NSE	+	+	+	+			
GFAP	-	-	-	-			
NT	-	-	-	-			
ER alpha	+	+	+	+			
ER beta	+	+	+	+			
Tph	+	+	-	w			
Socs-3	+	+	+	+			
AR	-	-	N/A	+			
G2R	+	+	+	-			
CRF	-	-	-	-			
GnRH	+	+	+	w			
POMC	-	+	+	-			
Gal	+	-	w	-			
Lep Receptor	-	+	+	w			
Agrp	+	+	+	+			
Cart	-	-	-	-			
NPY	-	-	+	+			
proGlu	-	w	w	-			
TH	+	-	+	-			
GHRH	-	+	+	+			
Avp	+	+	w	w			
proTRH	-	-	-	-			
Ucn	-	-	-	-			
MCH	+	N/A	+	+			
orexin	-	-	-	-			
DAT	strong	-	w	-			
CRFR1	-	-	-	-			
CRFR2	-	-	-	-			
Aromatase	-	-	-	strong			
GnRH Receptor	-	-	-	-			
Insulin receptor	+	+	+	+			
Oxytocin	+	+	+	+			
New-1	-	-	-	-			
New-2	-	-	-	-			
New-4	-	-	+	-			
GHS-R	N/A	N/A	N/A	-			
Leptin							
som							
NTR	+	w	N/A	-			
mc3R							
mc4R	N/A	N/A	N/A	-			
NPY-Y1							
NPY-Y2							
CRLR	N/A	N/A	N/A	-			
Ghrelin	+	+	N/A	+			
Ghrelin variant	+	-	N/A	-			

The following abbreviations will have their standard scientific abbreviations: T-Ag, Large T-antigen; NSE, neuron-specific enolase; GFAP, glial fibrillary acidic protein; SNTX, syntaxin; ER, estrogen receptor; AR, androgen receptor; LepR, leptin receptor b; Glp-2R (also G2R), glucagon-like peptide 2 receptor; SOCS-3, suppressor of cytokine signaling 3; NPY, neuropeptide Y; AGRP, agoutirelated peptide; POMC, proopiomelanocortin; CART, cocaine and amphetamine regulated transcript; MCH, melanin-concentrating hormone; Ucn, urocortin; NT, neurotensin; Gal, galanin; Orx, orexin; DAT, dopamine transporter; CRFR, corticotrophin-releasing factor receptor; proGlu, proglucagon; GHRH, growth hormone-releasing hormone; GnRH, gonadotropin-releasing hormone; GnRHR, gonadotropin-releasing hormone receptor; CRF, corticotropin-releasing factor; TRH, thyroid-releasing hormone; AVP, arginine vasopressin; OXY, oxytocin; Arom, aromatase; TPH, tryptophan hydroxytase; TH, tyrosine hydroxytase; TenM-1 (also New-1); TenM-2 (also New-2); TenM-3 (also New-3); and TenM-4 (also New-4), Teneurins 1-4; GHS-R, growth hormone secratogue receptor; Lep, leptin; SOM, somatostatin; NTR, neurotensin receptor; MC3R, melanocortin receptor-3; MC4R, melanocortin receptor-4; NPY-Y1, NPY receptor Y1; NPY-Y2, NPY receptor Y2; CRLR, calcitonin receptor like receptor; nd, not done; na, not done; w, weak expression.

Table 2: Genes Regulated by TCAP-1 at 16 hours

Cluster	Gene	Affimetrix Probe No.	Acc No. GB	Function	Fold change
Growth/ Differentiation	GAS5	98530	AI849615	Growth arrest specific transcript	0.46
	SDPR	160373	AI839175	Serum deprivation response protein	0.57
	PPAN	160802	AA674812	Peter Pan homologue	0.62
	CD95	102921	M83649	Fas antigen	0.61
	CRD-BP	102627	AF061569	CRD-binding protein	0.59
	SSG1	160298	AW122012	Steroid sensitive gene 1	0.62
	DIP1/2	97353	AI837497	DAB2 interacting protein	0.68
	GBP3	103202	AW047476	Guanylate binding protein	0.63
	P202	161173	AV229143	202 interferon activatable protein	0.61
	CAII	103441	AI94248	Casein kinase II	0.61
	INI1B	99924	AW121845	Integrase interacting protein 1B	0.48
	MMP1	100484	X66473	Matrix metalloproteinase 1	0.55
	MMP10	94724	Y13185	Matrix metalloproteinase 10	0.59
	PTK7	92325	AI326889	Receptor protein tyrosine kinase	1.53
	P204	98466	M31419	Interferon activatable protein	1.85
	MKI67	161931	AV309347	Cell cycle protein regulator	1.70
	MOP3	102382	AB014494	Circadian rhythm regulator	1.57
	ST7	160591	AI504013	Suppressor of tumourigenicity	1.97
	GDAP10	94192	Y17860	Ganglioside induced diff. protein 10	1.62
Signalling/ Communication	ERK1	101834	Z14249	Mitogen activated protein kinase	0.64
	ALK3	92767	D16250	Bone morphogenic protein receptor	0.60
	BMP4	93456	L47480	Bone morphogenic protein-4	0.52
	IL1R	93914	M20658	Interleukin 1 receptor	0.60
	GR	98818	X04435	Glucocorticoid receptor	0.66
	BARK1	104270	AA982714	β adrenergic receptor kinase 1	0.61
	CAMIII	92631	M19380	Calmodulin III	0.53
	PCDHγ	160976	AA222943	protocadherin γ	0.42
	AKAP95	95001	AB028920	A kinase anchor protein 95	0.60
	TTF-1IP	161019	W41560	TTF-1 interacting peptide	0.50
	CREMβ1	100533	M60285	cAMP-responsive element modulator	1.61
	AKAP8	161088	AV171460	A kinase anchor protein 8	1.58
	PDE6A	100696	X60664	cGMP Phosphodiesterase α	1.68
	INOS	104420	U43428	Inducible nitric oxide synthetase	1.50
	FNBX	92754	D49920	Ferredoxin-NADP reductase	1.61
	SLC6A4	161695	AV230927	Serotonin transporter	1.53
	CLCN3	94465	AF029347	Chloride channel protein 3	1.66
Processing	ARF1	95156	AI1853873	ADP ribosylation factor 1	0.63
	CLM2-B	93492	AB013469	Cytohesin-2	0.63
	YIPID	99675	AI839766	Rab-mediated membrane transport	1.88
	RAB10	160149	AI841543	Ras oncogene homologue	1.62
	GP25L2	100074	AW046723	gp25L brings cargo forward from ER	1.53
	AP4S1	104561	AI847561	Adaptor related protein complex	1.52

The change in expression levels is indicated relative to the untreated control cell for the same time period of 16 hours. Values >1.5 fold or <0.70 fold were considered significant.

FULL CITATIONS FOR REFERENCES REFERRED TO IN THE SPECIFICATION

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